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DNA-PROTEIN RELATIONS DURING MICROSPOROGENESIS OF TRADESCANTIA.

By

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With 6 figures in the text.

(Eingegangen am 12. Juni 1951.)

Introduction.

Only recently has an analysis of the relationship of desoxyribose nucleic acid (DNA), and proteins within the individual nucleus become possible. Historically this work derives from the pioneer researches of MIESCHER ('72), who isolated nuclear material from pus cells and from fish sperm by subjecting them to digestion with proteolytic enzyme preparations to remove cytoplasmic materials, as well as from the work of KOSSEL ('84), who isolated and characterised the protein components of nuclear extracts. It was later shown by ALTMANN ('89) that MIESCHER's nuclein could be split into an acid component rich in phosphorus, to which he gave the name nucleic acid, and into a more basic component which he showed to be a protein or mixture of proteins, rich in nitrogen.

Since then, LEVENE, MIKESKA and MORI (1930) have shown the nucleic acid component to be DNA, which is the chromosomal component revealed by Feulgen's nucleal reaction (FEULGEN and ROSSENBECK, 1924). The studies of AVERY, MCLEOD and MCCARTY (1944), BOIVIN, DELAUNY, VENDRELY and LEHOULT (1945), as well as the work of HOLLAENDER and CLAUS (1936), HOLLAENDER and EMMONS (1941), and others, indicate that DNA is concerned in genetic phenomena, and that it may be a major constituent of the presumed hereditary substance. About the protein constituents of nuclei, on the other hand, comparatively little is known.

[1] I wish to express my deep appreciation to Professors FRANZ SCHRADER and ARTHUR W. POLLISTER for their guidance and criticisms throughout the course of this work. — Thanks are also due to Professor C. L. HUSKINS of the University of Wisconsin for his kindness in supplying the stock plants, and to the Botany Department of Columbia University which generously provided greenhouse facilities. — The work was done while the author was a MCCALLUM Fellow of Columbia University.

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It is evident that biochemical procedures deal with millions of cells or isolated nuclei, and therefore are only capable of giving an overall picture of a nucleus. With the introduction of microscopic photometric procedures analysis at the cytological level has become possible. These methods have their origin in (1) the work of CASPERSSON (1936) who made use of the characteristic absorption phenomena of nucleic acids and proteins in the ultra-violet region, and (2) more recently the work of POLLISTER and RIS (1947), in which chemical reactions were employed to produce insoluble coloured derivatives which possess characteristic absorption maxima in the visible region of the spectrum.

The work reported in this paper, utilising photometric procedures, is concerned with the relationship between DNA and the proteins of nuclei during the development of the male germ cells of *Tradescantia paludosa*. This material was chosen since it has been the subject of many cytological studies, and the nuclei lend themselves very well to the techniques of microscopic photometric analysis.

Materials and Methods.

From stock material kindly supplied by Professor C. L. HUSKINS of the University of Wisconsin, clones of *Tradescantia paludosa* were established by vegetative means, all plants being kept under greenhouse conditions.

a) Fixation.

Periodically buds were removed into Carnoy's acetic alcohol (1:3) fixative. The buds were then opened to facilitate penetration of the fixing fluid. After one hour, an anther was rapidly removed from each bud and smeared in aceto-orcein, the stage of development of the microspores being recorded. If an anther proved to be sterile the bud was discarded. The timing of gametogenesis was found to be approximately the same as that reported by SAX and EDMONDS (1933) for *Tradescantia reflexa*. After a total fixation time of four hours[1] the buds were washed several times with absolute alcohol, cleared in benzene, embedded in paraffin and sectioned at various thicknesses. One bud was selected arbitrarily and used as a control; that is, sections of it were mounted on all subsequent series of slides and used as a check on the reproducibility of the staining reactions. Sections of several buds were mounted on the same slide. In all cases control sections gave identical results within the limits of experimental error.

b) Apparatus.

The techniques of photometric analysis have been presented in detail by POLLISTER and RIS (1947), DI STEFANO (1948), RIS and MIRSKY (1949), POLLISTER and MOSES (1949), LEUCHTENBERGER (1950), SWIFT (1950) and by POLLISTER (1950, 1951).

Essentially, the apparatus used in this study was identical with that described by LEUCHTENBERGER (1950) and SWIFT (1950). However instead of an R.C.A. 931 A electron multiplier tube, a G.L. 1P21 phototube (selected for high sensitivity)

[1] A preliminary experiment indicated that identical results were obtainable from material fixed for 4 or for 24 hours.

was used to measure transmissions. The light source was an AH4 Mercury vapour lamp. By means of the requisite filters (see below) essentially monochromatic illumination was achieved, the wavelengths isolated being close to the absorption maxima of the coloured compounds produced in the tissue sections. The optics consisted of a Zeiss NA 1·4 aplanatic condensor, Zeiss apochromatic oil immersion objective 2 mm, NA 1·3 and a Spencer 10× compensating ocular.

c) Staining.

In order for a staining reaction to be of use in chemical cytology, it must fulfill the following criteria: (1) the reaction must be capable of being standardised and it must be reproducible within narrow limits when carried out under standardised conditions.

(2) it is extremely important that the method fulfills the criterion of specificity, that is, a positive reaction is obtainable only in the presence of the compound under investigation and never, if it be absent.

(3) that there be evidence of a correlation between the results of *in vitro* experiments and the results obtained in tissue sections.

The desoxyribose nucleic acid (DNA) content was estimated in terms of the Feulgen nucleal reaction. Protein measurements were based on the Millon reaction, as well as staining with the dye Fast green.

1. DNA-Feulgen.

The Feulgen reagent was prepared according to the method of STOWELL (1945) and slides were stained for two hours at room temperature following optimal hydrolysis in 1N HCl (12 mins) at 60° C. All slides were stained in the same batch of reagent, though staining was carried out at different times.

That the Feulgen reagent gives reproducible results when used under standardised conditions is evident from the work of LEUCHTENBERGER (1950) as well as from the control sections used in the work reported here, all of the latter giving identical results within the limits of experimental error [see also POLLISTER (1951)]. That the Feulgen reaction is specific, is shown by the fact that when DNA is removed by a chemical agent such as trichloracetic acid (SCHNEIDER 1945), the tissue sections give a negative Feulgen reaction.

The Feulgen reaction was measured as the absorption of the 546 mμ line isolated from an AH4 lamp by means of a Farrand interference filter (peak transmission 549 mμ, 12 mμ half band width).

2. Millon reaction.

The Millon reaction standardised for cytochemical purposes by POLLISTER and MIRSKY (1946) was used for the demonstration of proteins in tissue sections. The reagents for the demonstration of total protein, and non-histone protein, were prepared as described by POLLISTER (1950)[1].

The *in vitro* specificity of the Millon reaction for tyrosine and tryptophane in proteins has been demonstrated by FOLIN and CIOCALTEU (1927). According to GIBBS (1926) the colour is produced by the formation of a nitroso compound. Specificity has also been confirmed cytochemically by the observation that salmon sperm nuclei which contain tyrosine-free protamines yield a negative Millon reaction (POLLISTER unpublished). The reproducibility of the Millon reaction

[1] The final concentration of trichloracetic acid in reagent B, should be 1·8 Molar instead of 0·9 Molar.

has been demonstrated by absorption measurements on thyroid colloid by POLLISTER and MIRSKY (1946) and in nucleoli of maize pollen mother cells by POLLISTER and RIS (1947), also in the present work by measurements on the control sections referred to earlier.

The colour developed was measured as the absorption of the 365 mμ line isolated from an AH4 lamp by a Corning glass filter No. 5840, 2·4 mm thick.

3. Acid Staining.

It is clear from the studies of CHAPMAN, GREENBERG and SCHMIDT (1927) and from the researches of FRAENKEL-CONRAT and COOPER (1944) that the amounts of acid dyes bound at low p_H are stoichiometrically related to the amounts of free basic groups present in various proteins. In the present study, a 0·1% solution of Fast green (Fast green F.C.F. National Aniline Division, dye content 96%) in N/10 HCl was prepared and used as described by SCHRADER and LEUCHTENBERGER (1950).

The dye bound was measured as the absorption of the 623 mμ line isolated from an AH4 lamp by means of a Wratten No. 26 filter.

Reference to Table 1 indicates that Fast green staining is reproducible under he conditions used in this study.

Table 1. *Reproducibility of Fast green staining.*

Slide No.	Material	No. of Nuclei Measured	Mean E_{623}	Concentration *
1	Bud scale epidermis 7·3 microns	17	1·048	0·144
2	Bud scale epidermis 7·3 microns	20	0·990	0·136
3	Bud scale epidermis 7·3 microns	11	1·033	0·142
4	Bud scale epidermis 7·3 microns	9	1·002	0·138

* see page 5 for scheme of computation.

4. Basic Staining.

Basophilia was followed throughout microspore development by the use of Azure B according to the method of FLAX and HIMES (1951). Also experiments were performed to determine the effects of (1) deamination procedures[1] and (2) of digestion with pepsin in hydrochloric acid[2] on the intensity of Fast green staining. Since these experiments were of a preliminary nature, no actual measurements are reported at the present time.

[1] A modification of the method of VAN SLYKE (1914). Reagent A, 4% $NaNO_2$; reagent B, glacial acetic acid. Slides were treated for $1^1/_2$–2 hrs. at 18° C. in a mixture of 50 ml. reagent A, and 5 ml. reagent B. Control slides were left for the same time in 50 ml. distilled water plus 5 ml. of reagent B. (MONNÉ and SLAUTTERBACK, 1951, have recently described the use of the VAN SLYKE method to determine chemical acid dye staining. Their method differs in several details from the above.)

[2] Parke Davis pepsin (1:3000) in 0·01 N HCl, see SCHRADER and LEUCHTENBERGER (1951), for detailed procedure.

d) Computation.

It is self evident that photometric measurements carried out on stained tissue sections yield data which refer not to the absolute content of nucleic acid or proteins present in nuclei, but rather to the amount of coloured substance produced by the chemical reaction employed. That the amount of colour produced, or dye bound, is proportional to the nucleic acid or protein content, is conclusively demonstrated by the data of RIS and MIRSKY (1949), SWIFT (1949), PASTEELS and LISON (1950) and LEUCHTENBERGER, VENDRELY and VENDRELY (1951).

The following procedure was adopted in order to measure the amounts of coloured substance produced in tissue sections by the reactions employed. The lamp diaphragm was stopped down to 1·0 mm. and the condenser diaphragm closed to 10 mm. The scanner diaphragm was adjusted so as to allow an image area of 1·3, 2 or 4 mm. diameter taken through the centre of a nuclear image to fall on the sensory surface of the phototube, a diameter of 1·3 mm. corresponding to an object size of 2·25 μ. Nuclear diameters were measured by means of a calibrated ocular micrometer mounted on the scanner unit. Readings were recorded as the deflections on a Weston micro-ammeter with a maximum deflection of 10 microamperes, readable to 1/100 micro-ampere. Background readings were taken through optically empty regions of the slide, the fraction T/B giving the transmission in percent. The transmission data were converted into extinction values ($\log_{10}$ 1/T).

In all cases the appropriate blanks were prepared, that for the Feulgen reaction representing a measure of the non specific light loss, which was found to be negligible. The blanks for the Millon reaction were found to be somewhat higher; the mean value (calculated from measurements obtained from several slides), was an E_{365} of 0·035 for the total protein and E_{365} of 0·040 for the acid insoluble protein. The extinction values were therefore corrected by subtraction of the appropriate blank. Protein determinations were also corrected for cytoplasmic absorption in the case of measurements on intact nuclei. From the corrected extinctions, the content of DNA-Feulgen and of protein (as Millon and Fast green) was computed for each nucleus as follows.

As discussed by POLLISTER and RIS (1947) and RIS and MIRSKY (1949), if given the extinction of a solution where concentration and thickness are known, one may calculate the concentration of an unknown from its extinction value by substituting in the following equation (1) derived from the BEER-LAMBERT Law.

$$C_2 = \frac{d_1 \cdot E_2}{d_2 \cdot E_1} \cdot C_1 \qquad (1)$$

where C_1 and C_2 are the concentrations respectively of the standard and unknown, d_1 and d_2 the respective thicknesses of the absorbing layer (Mean Optical Path), E_1 is the extinction of a standard, and E_2 the measured extinction. If one is only concerned with relative values (as is the case here), then equation (1) may be reduced to the expression (2)

$$C = \frac{E_2}{d_2}. \qquad (2)$$

If the value C in equation (2) is multiplied by the nuclear volume, an expression (in arbitrary units) of the amount of absorbing material is obtained for each nucleus considered. For an absorbing sphere, the thickness (d_2 in equation 2) is equal to the Mean Optical Path (MOP) of the sphere, whereas for a thin section of a sphere it is equal to the section thickness (see also KORSON 1951). In cases where thin sections of nuclei were used, the thickness was determined by means of measurements made with a Bausch and Lomb Phase-Contrast microscope.

The MOP of an entire nucleus may be calculated by converting the volume, taken as a sphere, to that of a cylinder of identical cross-sectional area and solving for h where h = height of cylinder

$$\text{thus } 4/3\pi \cdot r^3 = \pi \cdot r^2 \cdot h$$
$$\text{this reduces to } h = \text{MOP} = 4/3r. \tag{3}$$

If instead of a whole sphere, a cylindrical plug through the centre is measured then a different formula must be used to calculate the MOP

$$d = \text{MOP} = \frac{4 \cdot F \cdot r^3}{3 \cdot R^2} \tag{4}$$

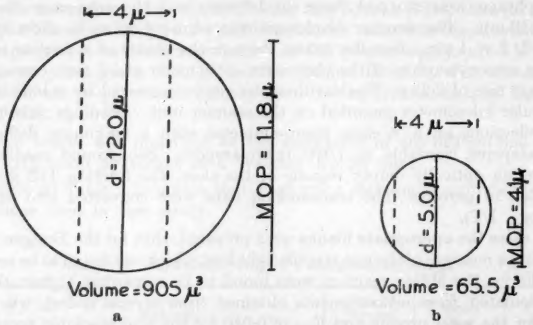


Fig. 1 a and b. Diagram illustrating methods of computation. Explanation in text.

Computation of Relative Amounts assuming an E of Plug of 0.300.

	Fig. 1 a	Fig. 1 b
Method (1)	23·0	4·8
Method (2)	22·6	3·9
Difference	2·0%	23·0%

where R = radius of cylinder, r = radius of sphere, F = the fraction of nuclear volume included in the cylinder and may be calculated from the expression

$$F = \frac{r^3 - (r^2 - R^2)^{3/2}}{r^3}. \tag{5}$$

In cases where equation (4) applied, the smallest nucleus in the sample was selected, and the amount of absorbing substance computed in two ways; (1) the MOP was calculated and substituted for d_2 in equation (2), and (2) the diameter of the nucleus was substituted for d_2. If the results obtained differed by no more than 10%, computations of the MOP were then omitted, for the error introduced by such a procedure is, in the case of the smallest nucleus, the maximum possible for the sample (figure 1 illustrates these methods of computation assuming an extinction of 0·300).

Provided that this procedure was possible, then the following formula (6) was used, where it can be seen that the thickness need not be considered, the only assumption being the previous one, that MOP = $2r$.

$$\textit{Then } \frac{E}{2r} \cdot 4/3\pi \cdot r^3 = \textit{amount} \qquad \text{(from 2 above)}$$
$$\text{this reduces to } E \cdot 2/3\pi \cdot r^2. \tag{6}$$

Certain nuclei are better regarded as prolate spheroids than as spheres, as in the case of the generative nuclei. In such cases, the volume is given by the expression $4/3\pi \cdot r_1 \cdot r_2^2$ where r_1 and r_2 are the major and minor radii. Substituting in (6) we get:

$$amount = E \cdot 2/3\pi \cdot r_1 \cdot r_2. \qquad (6a)$$

It was desirable to compare data obtained from measurements on intact nuclei in thick sections with data obtained from thin sections of nuclei. The following experiment indicates such a comparison to be valid. Intact nuclei were selected from bud scale epidermis[1] sectioned at 20–30 microns; the thin sections of nuclei were provided by 7·4 μ sections of the same bud scale material. Sections of each thickness were mounted on the same slide and stained with the Feulgen reagent.

Table 2. *Comparison of measurements on intact nuclei and thin sections.*

Thickness	Nuclear Type	No. of Nuclei Measured	Amount in Arbitrary Units
20 microns	Class I	18	38·45 ± 0·78
	Class II	7	51·60 ± 0·93
7·4 microns	Class I	7	37·60 ± 0·75
	Class II	8	51·90 ± 0·90

From Table 2 it can be seen that the results obtained do not differ significantly and one may therefore use either slices of nuclei or intact nuclei. Nuclei must however, be homogenous with respect to the distribution of the Feulgen-positive material if measurements on slices of nuclei are to be compared to similar measurements on intact nuclei. It is also obvious that, in the bud scale epidermis, there may exist populations of nuclei. In the present case two classes were found, one of which contained approximately the diploid amount, while the other contained an amount intermediate betweeen the diploid and two times this value.

Results.

a) Cytological Studies.

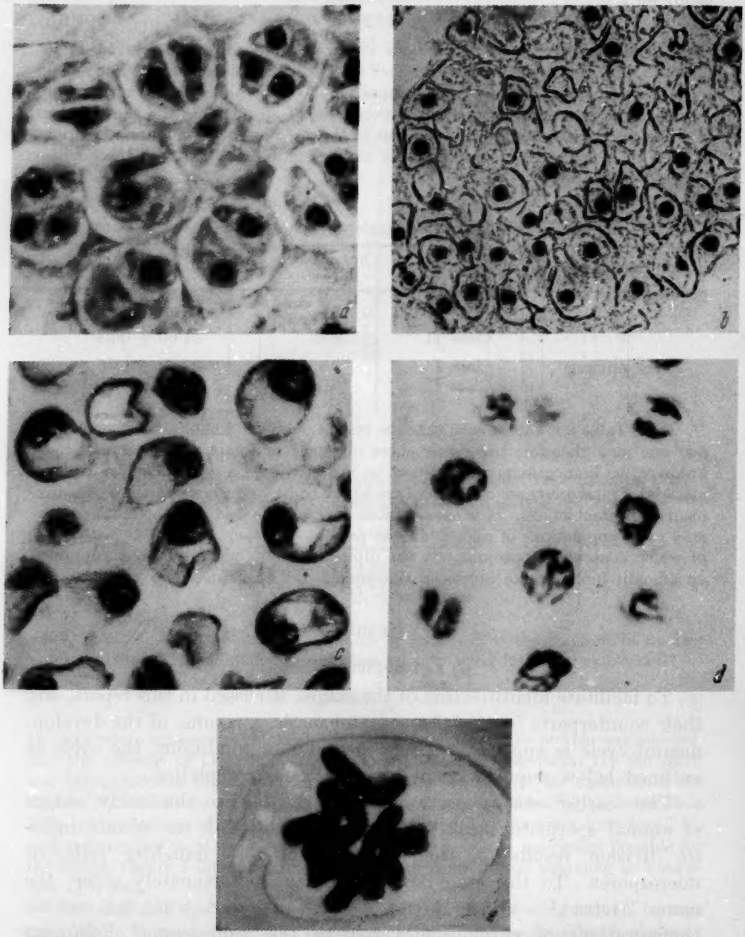
To facilitate identification of the stages discussed in this report, and their counterparts in animal spermatogenesis, a resume of the developmental cycle is appended. Under greenhouse conditions, the cycle as outlined below requires about two weeks for completion.

The earlier stages are essentially similar to the early stages of animal spermatogenesis. As in animal material, the second meiotic division results in the formation of four daughter cells, or microspores. To this stage, botanists have unfortunately given the name "tetrad[2]" which should not be confused with the meiotic configurations of chromosomes bearing the same name. Following

[1] The bud scale epidermis was used as the representative of mature somatic tissues in all subsequent experiments.

[2] SHARP (1933) has suggested the term "quartet" be used instead of tetrad, but it has not been accepted into common usage.

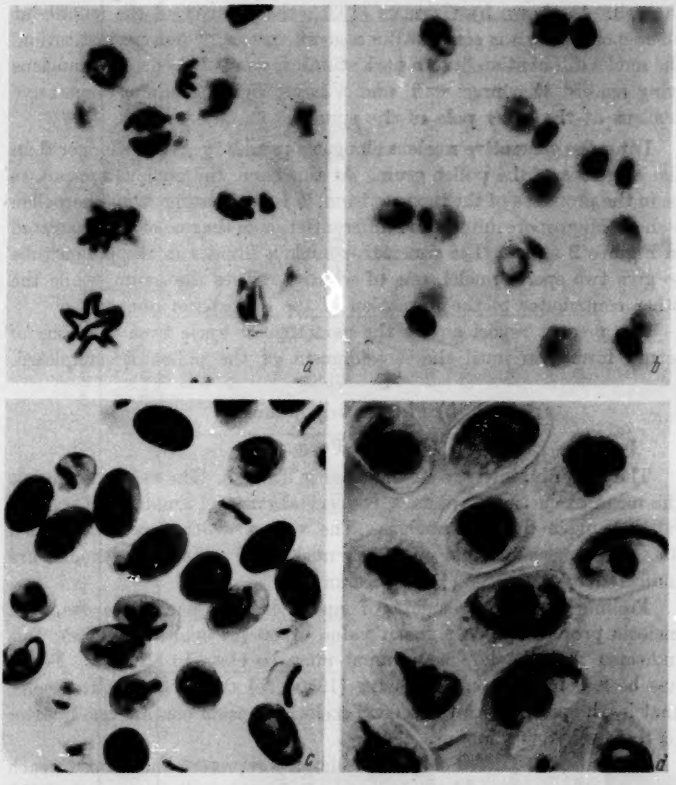
tetrad formation, there is a long interphasic period in which three morphological stages may be identified.



Figs. 2 *a*—*e*. Stages in microsporogenesis. *a* — tetrad stage; Azure B; *b* — early microspores; Feulgen; *c* — late microspores; Safránine; *d* — prophase of microspore division; Feulgen; *e* — a typical metaphase plate showing haploid number of chromosomes; Feulgen.

In *Tradescantia*, the four daughter cells separate from each other early in the interphasic period; this stage I have designated as "early

microspores" for convenience of reference (see Figure 2). Later microspores show the nucleus to be displaced to one end of the cell by the development of a large vacuole. Prior to microspore mitosis, the nucleus



Figs. 3*a*—*d*. Stages in microsporogenesis continued. *a* — microspores in mitosis; Feulgen; *b* — pollen grains with generative and vegetative nuclei; Feulgen; *c* — mature pollen grains, note cytoplasmic basophilia; Safranine; *d* — pollen prior to anthesis showing elongate generative nuclei; Feulgen.

is again found to occupy the central part of the microspore (bounded by vacuoles). These later stages of microspore interphase will hereinafter be referred to as "mid microspores" and "late microspores" respectively, and are illustrated in Figure 2. It is during this latter part of the interphase that microspore growth occurs.

Towards the end of the interphase, the microspores show one wall to be slightly flattened. This corresponds to what may be termed the inner wall with respect to orientation at the tetrad stage. Division of the microspore is, under normal conditions, along the short axis of the cell. As shown by GEITLER (1935), the polarity of the spindle at microspore mitosis is constant for a given species. Upon reconstitution, the nuclei differentiate into a dark staining, compact generative nucleus lying against the inner wall, and a large, lightly staining vegetative nucleus at the outer pole of the spindle.

Later the generative nucleus elongates until it is actually longer than the long axis of the pollen grain. At this time, the nucleus appears to be in the prophase of the next division; it is at this stage that the pollen is shed. Microspore mitosis and differentiation of the nuclei are illustrated in Figures 2 and 3. The generative nucleus divides in the pollen tube to give two sperm nuclei, one of which fertilises the ovum, while the other contributes to the formation of the endosperm nucleus.

The present report covers the gametogenic cycle from the time of tetrad formation until the development of the pollen is completed.

b) Photometric Studies.

1. DNA-Feulgen.

The data obtained are summarised in Table 3. The values represent the mean extinctions, volumes, concentrations and amounts of regenerated Feulgen dye per nucleus. The values which represent pooled data from measurements on intact nuclei and on slices of nuclei are indicated by a dagger (†) in column 4.

Preliminary measurements of nuclei in the pachytene stage of meiotic prophase gave a mean value of 60 (in arbitrary units); this indicates that the haploid amount must be close to 15 units. For it has been demonstrated by SWIFT (1950a, b) that the daughter cells, that is, the products of the second meiotic division contain one quarter of the pachytene amount.

The ratios of DNA-Feulgen in column 8 represent the ratios of each stage to this presumed haploid amount. On this assumption, the DNA content of nuclei taken from the bud scale epidermis is seen to vary between the diploid value and two times this amount. Cytological examination of the tetrad stage revealed that the buds had been fixed some time after the onset of interphase and indicated that there had been further development of the microspores beyond the early tetrad stage. In agreement with this fact, the DNA content of these nuclei shows an increase of about 50% over the theoretical value. It is of interest that the present findings agree quite well with the data of

Table 3. *DNA-Feulgen Content of Nuclei.*

Bud	Stage	No. of Nuclei Measured	Mean Extinction E_{546}	Mean Nuclear Volume Microns³	Mean Concentration	Mean Amounts in Arbitrary Units	Approximate Ratio Assuming $n = 15$
1	Bud scale Epidermis I	25	0·902†	337	0·109	36·60 ± 0·73	2:1
	II	15	1·010†	425	0·122	51·75 ± 0·92	3:1
2	Bud scale Epidermis . . . . . (7·4 microns)	43	0·886	587	0·120	67·70 ± 1·4	4·5:1
3	Late Tetrad (intact nuclei) . .	20	1·379	84	0·252	21·18 ± 0·55	1·4:1
4	Early Microspores (intact nuclei)	27	1·110	146	0·162	23·60 ± 0·62	1·6:1
5	Mid Microspore . . . . . (7·3 microns)	21	0·332	579	0·044	25·38 ± 2·16	1·7:1
6	Late Microspores . . . . . (7·3 microns)	30	0·982	685	0·135	64·40 ± 0·97	4:1
7	Pollen Telophase Generative . . (intact nuclei)	27	0·827	190	0·163 *	31·00 ± 0·91	2:1
	Vegetative . . . . . (7·3 microns)	17	0·372	539	0·050	27·10 ± 1·64	2:1
8	Pollen Interphase Generative . (intact nuclei)	32	1·150	135	0·197	26·60 ± 0·85	2:1
	Vegetative (intact nuclei) . . .	32	0·611	519	0·059	30·60 ± 1·25	2:1
9	Late vegetative (intact nuclei) .	21	1·080	350	0·122	42·60 ± 1·37	3:1

† pooled data. * MOP considered.

SCHRADER and LEUCHTENBERGER (1949) obtained from cytologically similar material.

Data from Table 3 are presented in block-diagram form in Figure 4.

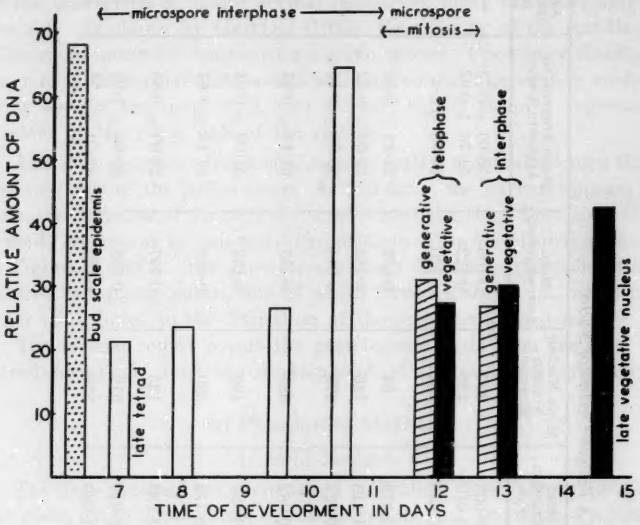


Fig. 4. The DNA content of nuclei during microspore development.

2. Proteins.

(a) The Millon Reaction.

The results obtained from absorption measurements of the Millon reaction are presented in summary form in Table 4. Only the values for the total protein and acid insoluble protein are given, the values for the acid soluble protein may be obtained by subtraction. The results are depicted in block-diagram form in Figure 5 together with the mean nuclear volume of each stage.

(b) Acid Staining.

Data of a preliminary nature obtained from microscopic photometric measurements of Fast green stained nuclei are summarised in Table 5, and are also presented in Figure 6 together with the corresponding Millon values[1].

[1] Since the nuclear volumes for each tissue varied somewhat, concentrations were used in Figure 6 rather than amounts of proteins.

Table 4. *Protein (Millon) Content of Nuclei.*

Bud	Stage	Type of Protein	No. of Measurements	Mean[1] Extinction E_{555}	Mean Volume Microns^3	Concentrations	Amount in Arbitrary Units	Total Protein/DNA Ratio
1	Bud scale Epidermis (7·3 microns)	Total	22	0·272	520	0·037	19·87 ± 0·54	I 0·54 II 0·38
		Acid insoluble[2]	20	0·051	449	0·007	3·06 ± 0·21	—
2	Bud scale Epidermis (7·4 microns)	Total	25	0·136	703	0·018	12·40 ± 0·89	0·18
		Acid insoluble .	23	0·060	612	0·008	4·95 ± 0·38	—
3	Late Tetrad (intact nuclei)	Total	22	0·197*	106	0·044	4·65 ± 0·11	0·22
		Acid insoluble .	23	0·066†	238	0·010	2·34 ± 0·16	—
4	Early Microspores (intact nuclei)	Total	25	0·259	147	0·039	5·74 ± 0·15	0·24
		Acid insoluble .	21	0·088	168	0·013	2·14 ± 0·15	—
5	Mid Microspores (7·3 microns)	Total	19	6·116	760	0·015	11·90 ± 0·82	0·47
		Acid insoluble .	18	0·082	670	0·010	7·21 ± 0·51	—
6	Late Microspores (7·3 microns)	Total	35	0·207	742	0·028	21·05 ± 0·86	0·33
		Acid insoluble .	21	0·091	595	0·013	7·25 ± 0·55	—
8	Pollen Interphase Generative (intact nuclei)	Total	25	0·274	152	0·049	7·47 ± 0·37	0·28
		Acid insoluble .	24	0·093	154	0·016	2·48 ± 0·21	—
	Vegetative (7·3 microns)	Total	21	0·230	664	0·031	20·55 ± 0·43	0·67
		Acid insoluble .	23	0·094	515	0·013	6·45 ± 0·54	—

[1] after subtraction of blanks. [2] protein insoluble in the reagent used for the removal of histones (see POLLISTER 1950). * MOP considered. † pooled data.

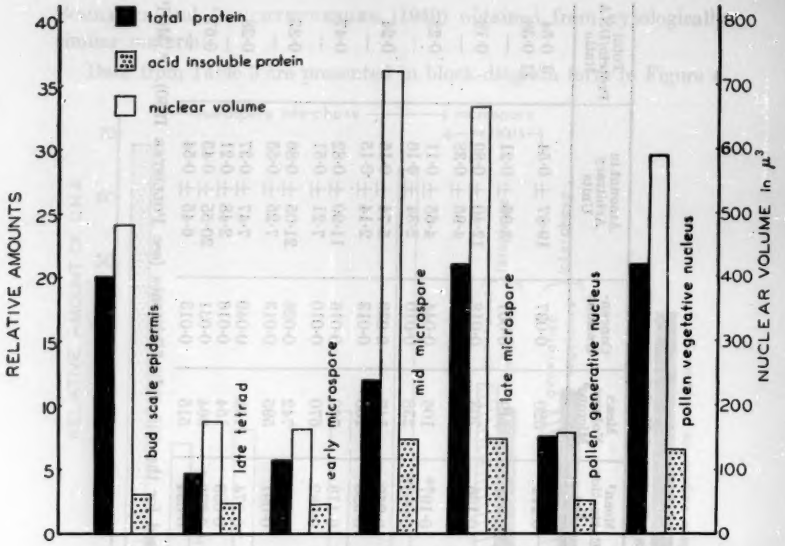


Fig. 5. The protein (Millon) content of nuclei during microspore development.

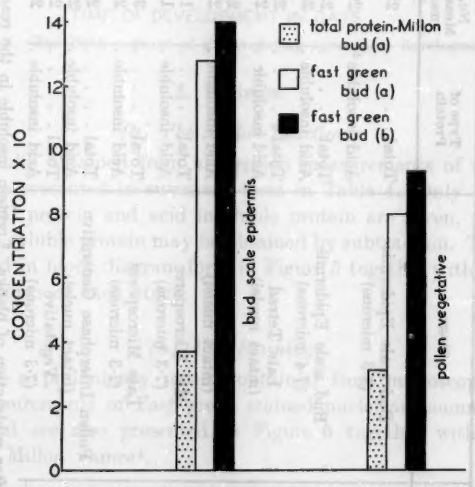


Fig. 6. The protein content of nuclei as determined by the Millon reaction and by staining with Fast green.

The DNA content may be related to the protein content of nuclei by means of a ratio (column 8, Table 4) which may be computed from the data summarised in the preceding tables. It should be pointed out that the values obtained are purely relative and give no indication of the relationship in absolute terms. The changes in ratio do, however, provide a valid measure of the relative rates of DNA and protein synthesis during microsporogenesis.

Table 5. *Protein (Fast green) content of Nuclei.*

	Stage	No. of Nuclei Measured	Mean Extinction $E_{m\mu}$	Concentration	Mean Volume Microns³	Amount in Relative Units
Bud (a)	Bud scale Epidermis (7·3 microns)	25	0·935	0·128	401	51·4
	Pollen Vegetative	25	0·580	0·080	605	48·0
Bud (b)	Bud scale Epidermis (7·3 microns)	37	1·019	0·140	454	63·5
	Pollen Vegetative	25	0·675	0·093	514	47·8

Discussion.

a) DNA-Feulgen.

By the use of chemical procedures, Boivin, Vendrely and Vendrely (1948), and others, were able to determine the DNA content of nuclei isolated from various mammalian tissues. The DNA content of nuclei has also been determined photometrically, in tissue sections, by Ris and Mirsky (1949), and other workers (for a summary of the early papers, using these two methods, see Pollister, Swift and Alfert 1951). Both techniques have been used simultaneously — on identical material by Ris and Mirsky (1949), and by Leuchtenberger, Vendrely and Vendrely (1950).

The significant conclusion which may be drawn from the above work is that, for a given species, there exists a characteristic amount of DNA per the adult somatic nucleus. In cases where chromosome counts have indicated diploidy, this value has been found to be twice that obtained for sperm cells. Data obtained by photometric means also revealed the existence of nuclei possessing two and four times the diploid amount in organs such as the liver of rodents, where it has been shown that polyploid classes exist (e. g. by Biesele, 1944). Thereby is furnished an explanation of the intermediate values obtained for the same organs by chemical means which — it is obvious — can give only average values.

In the case of plants however, less information is available. The evidence of SCHRADER and LEUCHTENBERGER (1949) and of SWIFT (1950b) indicates that the DNA content of somatic nuclei apparently tends to depart from the diploid value, the final values being somewhat different for the various tissues considered. Both authors point out that the values obtained may be accounted for on the basis of polyteny, or rather, reduplications of the chromonemata. This does not mean that the results of these workers are in agreement. The data of SWIFT lends itself to the inference that reduplications of the chromonemata are synchronised and constitute a process rather similar to endomitosis. The occurrence of DNA in multiples of the diploid amount, and the lack of intermediate values in non-dividing tissues support such a conclusion.

The findings of SCHRADER and LEUCHTENBERGER indicate a lack of synchronisation in the reduplication process — such as was also described by HUSKINS and STEINITZ (1948) in the case of *Rhoeo*. This leads to the unavoidable conclusion that in mature, non-dividing tissues, partial reduplication of the chromonematal set does occur. With respect to the bud-scale epidermis, my findings also indicate a partial reduplication of chromonemata, and thus are in agreement with the results obtained by SCHRADER and LEUCHTENBERGER.

From the data presented here, it follows that (a) the DNA content of developing microspores increases during the long interphasic period; (b) that this increase is rather slow during early interphase and extremely rapid just prior to microspore prophase, attaining a value four times the haploid value; (c) there is equipartition of DNA at microspore metaphase (generative and vegetative nuclei possessing identical amounts), and (d) there is a post mitotic increase in DNA content of the vegetative nucleus.

In a recently published paper OGUR, ERICKSON, ROSEN, SAX and HOLDEN (1951) have reported on the DNA content of nuclei during microsporogenesis of *Lilium longifolium*. Their findings are based on microchemical procedures which involve (1) the extraction of nucleic acids by means of a chemical agent — such as perchloric acid (OGUR and ROSEN, 1950), and (2) the assay of the extracts for DNA by means of the Dische reaction (DISCHE 1930) and by ultra-violet absorption measurements. The results obtained by these workers are presented in Table 6 together with comparable data obtained (by the present author) from photometric measurements on *Tradescantia*.

It is clear that my own findings, based on microscopic photometric measurements on *Tradescantia*, agree very well indeed with the results obtained by OGUR and co-workers for similar stages in *Lilium*.

Table 6. *A comparison of the DNA content of developing microspores as determined by photometric and microchemical methods.*

Photometric[1]		Microchemical[2]	
Stage[3]	Ratio	Stage	Ratio
Pachytene	4·0	Late meiotic microsporocyte . .	4·8
Late tetrad	1·4	Earliest microspore	1·0
Mid-microspore	1·7	Microspore just before mitosis . .	2·0
Pollen-telophase	2·0	Microspore in early mitosis . . .	2·4
Late vegetative nuclei	3·0	Pollen at anthesis	3·6

[1] from Table 3. [2] from OGUR, ERICKSON, ROSEN, SAX and HOLDEN (1951). [3] stages in my material comparable to those of OGUR and co-workers.

It will be seen that the present findings and those of OGUR et alii, disagree with those previously reported by SWIFT (1950) inasmuch as they indicate that the generative and vegetative nuclei, though possessing a haploid chromosome set, contain the diploid amount of DNA. Also in that they indicate that there is a post mitotic increase in DNA content, not attributable — as claimed by SWIFT — to the build-up of the DNA content prior to the further division of the generative nucleus. The data of OGUR and his collaborators (1951) indicate that at anthesis the pollen grain must contain eight times as much DNA as is found in the very early tetrad nucleus. It is unfortunate that the morphology of the differentiating generative nucleus precludes further analysis by microscopic photometric means, and that microchemical procedures are only capable of yielding information regarding the total DNA content of the pollen grain; for, if one assumes with OGUR et alia that the post mitotic increase is shared by both nuclei then it follows that each *sperm* nucleus should contain the diploid amount of DNA. This is in conflict with genetical concepts unless one further assumes that synthesis of DNA prior to the first post zygotic division occurs in the gametes *before* fertilisation, or rather before the formation of pro-nuclei. If valid, then this would constitute a notable distinction between animals and plants, for the findings of ALFERT (1950) indicate that such is not the case at least in the mouse. It is hoped that studies on oogenesis will provide a partial answer to this problem. Until such studies have been completed, one cannot conclusively state whether or not microsporogenesis constitutes an exception to the "Constancy" hypothesis.

With respect to the fate of the vegetative nucleus the prevalent concept is that, sooner or later, it undergoes degeneration (see MAHESHWARI, 1949). From his work on *Crinum*, SUITA (1937) reports that the vegetative nucleus increases markedly in size during telophase-interphase and loses its Feulgen positive character.

It has been stated by SUITA (1937) that on maturation of the pollen, the vegetative nucleus becomes amoeboid in form, decreases in volume and eventually disappears (as observed by Feulgen staining). KOLLER (1947) claims that the vegetative nucleus of *Tradescantia* is in a fluid state during its passage down the pollen tube. He cites the work of SUITA as supporting his views on the lack of structural integrity in the vegetative nucleus. The work of ANDERSON and SAX (1934) and of JOHNSON and PECK (1937) contradicts these views, since both offer conclusive evidence for the existence of structure in the vegetative nucleus. Cytological examination of Feulgen stained preparations by the present author confirms the findings of these latter workers and also those of SUITA regarding the changes in volume and shape of the nucleus.

If one rejects the previously advanced hypothesis based on the condition of the late pollen grain with respect to DNA content, then one must postulate that the post mitotic increase in DNA content occurs only in the vegetative nucleus. That some increase occurs is beyond doubt, but this should not be interpreted as necessarily indicating that an increase to three times the amount present at telophase takes place. It should be pointed out that my data pertain to slightly immature pollen; in older material fragmentation of the vegetative nuclei was frequently observed. This process, together with the amoeboid nature of the nuclei, precluded further cytochemical analysis though qualitative studies suggested that there may indeed be a further increase in DNA content beyond the stage measured.

However it is apparent from the literature that qualitative considerations regarding nuclei of different size, shape, and intensity of stain are fraught with many pitfalls. Thus it has commonly been concluded that at telophase the vegetative nucleus contains much less DNA than the generative nucleus. It is clear from the present study as well as the previous work of SWIFT (1950b), that objective photometric measurements prove these views to be false.

Until further work has been done, the precise nature of the increase in DNA content beyond the amount present at microspore telophase must remain an open question.

b) Proteins.

From the data summarised in Table 4 and in figure 5 it may be inferred that there is a rapid increase in protein content during the attenuated microspore interphase. Thus the protein content increases by about one fourth from the time of tetrad formation until the separation of the microspores and by nearly 3 times during the period between this stage and the beginning of microspore prophase. The combined

protein content of the pollen grain represents an increase of about one-third over that of the microspore nucleus in late interphase. This latter increase may have occurred in early prophase, or immediately after division was completed.

On *a priori* grounds, it would appear that the data are suggestive of the existence of an unequal division of protein between the vegetative and generative nucleus. For if the protein content of the generative nucleus is compared with that of the late microspore, then it may be seen that the vegetative nucleus must receive twice as much protein as the generative nucleus. Such a conclusion is hard to justify, since it is clear from the DNA studies that equipartition of at least one major chromosomal constituent occurs. It is rather hard to visualise, on this basis, equipartition of the DNA and a concomitant unequal distribution of chromosomal protein if the protein is an integral structural component of chromosomes — as some studies have suggested (MAZIA, 1941; KAUFMANN et al., 1949).

From his studies, CASPERSSON (1940; 1950) has suggested that there is a progressive loss of nuclear protein during prophase. He has also concluded that a rapid synthesis of protein occurs during the restitution of the daughter nuclei. If my own findings are considered in the light of CASPERSSON's work, then it may be concluded that the increase in protein occurs immediately following the division of the microspore nucleus. The present work therefore offers further support for the hypotheses of CASPERSSON with respect to protein synthesis and, as will be seen, receives some support from observations of a qualitative nature — to be discussed in the following section.

From his extensive researches JACOBJ (1935) published evidence for a step-wise seriation of nuclear volumes in various mammalian tissues. This has recently been confirmed for the hemipteran insect *Arvelius* by SCHRADER and LEUCHTENBERGER (1950). They showed, in contradiction to the views of HERTWIG (1934), that this variation was independent of DNA content but was related to the protein content of the nucleus. The present study offers further support for these views, though it will be noted that microspore nuclei in late interphase and pollen vegetative nuclei present an apparent exception to this rule. In these cases it should be pointed out that the large, strongly Millon-positive nucleoli were often seen to be close to the nuclear membrane. So in many cases they were excluded from the portion of the nucleus measured, the net effect being to reduce the amount of protein as determined by absorption measurements. From Table 4 it is clear that the acid insoluble protein constitutes up to one-half of the total protein content, again the vegetative nucleus possessing twice as much as the generative nucleus.

From the Fast green data summarised earlier, it may be concluded that the somatic nuclei contain about one and a half times as many basic groups as do vegetative nuclei of the same bud. This may be interpreted as indicating that the bulk of the proteins in the vegetative nuclei are of a more acid character; this is evident when the Fast green data are compared with data from the Millon reaction for the same tissues (see Figure 6).

It is not inappropriate to mention qualitative studies bearing on this point. It would appear from the early work of MIESCHER ('72), and from the more recent researches of MAZIA (1941), and KAUFMANN (KAUFMANN, GAY and MCDONALD, 1949), that pepsin-hydrochloric acid mixtures effect digestion of cellular protein, attacking preferentially those of a less basic nature, though as KOSSEL (1906) pointed out, very basic proteins (histones) are also to some extent degraded *in vitro* by digestion with pepsin (see also DALY, MIRSKY and RIS, 1951).

It was noticed in the case of Fast green stained material that vegetative nuclei bound about the same amount of dye as the surrounding cytoplasm, whereas generative nuclei and the nuclei of somatic tissues were considerably more deeply stained. Slides were also stained in Fast green after being subjected to the action of a pepsin hydrochloric acid mixture made up and used according to SCHRADER and LEUCHTENBERGER (1951). It was found that although there had been a marked reduction in the amounts of dye bound, vegetative nuclei and cytoplasm showed about equal reduction, whereas generative and somatic nuclei were much less reduced in staining intensity. Similar results were also obtained following deamination over short periods of time, using the previously mentioned modification of the Van Slyke reagent.

On the basis of these findings, the tentative conclusion appears justified that much of the protein contained in vegetative nuclei differs in its chemical constitution from that contained in somatic and generative nuclei, the indication being that it is more acid in character.

c) General Considerations.

There are two periods during microspore development at which increase in size — denoting true growth occurs: (a) towards the end of microspore interphase and (b) just prior to or during the final development of the pollen grains. Both microspore nuclei and vegetative nuclei show prominent and strongly acidophilic nucleoli. By means of basic and acid staining (Azure B and Fast green respectively) it was possible to correlate with the onset of growth, pronounced basophilia and acidophilia in the cytoplasm. The cytoplasm of maturing pollen grains gives both reactions to a greater extent than in the case of the

earlier stage. These observations appear to be in keeping with the findings of the CASPERSSON school relating to cellular growth processes (CASPERSSON 1947, 1950).

During the early growth of the microspore the tapetal cells undergo degeneration and discharge their contents into the locular cavity, where the tapetal nuclei may be seen in various stages of degeneration. At this time an increase in cytoplasmic basophilia becomes apparent. PAINTER (1943) assumes from a similar observation in *Rhoeo* that the tapetal material contributes to the growth of the developing microspore. He further suggests that this constitutes a case comparable to the developing oocytes of *Drosophila* (PAINTER and REINDORP 1939).

The condition found in the pollen grain affords a unique example of the phenomenon of differentiation. The work of GEITLER (1935), SAX (1937), and LA COUR (1949) suggests that in *Tradescantia* differentiation may be closely correlated with the polarity of the spindle. GEITLER has shown, as stated earlier, that for a given species the polarity is constant. SAX (1937), and LA COUR (1949) have shown that upon disturbance of polarity by experimental means or natural causes, differentiation does not occur; KOLLER (1943) obtained similar results in his irradiation studies. Since the generative nucleus is cut off from the rest of the cell by the cell plate, then perhaps nuclear-cytoplasmic relations are important in the differentiation process. Support for such a conclusion is afforded by mal-orientation of the spindle. Here each cell has a comparable amount of cytoplasm, whereas normally the generative nucleus is enclosed in a cell containing very little cytoplasm. This, moreover, stains differently from the rest of the cytoplasm and presents a more hyaline appearance. These observations suggest at least a qualitative difference between the cytoplasms of the two cells. At present it is still impossible to be certain whether the difference between the nuclei with respect to protein content is the result of an earlier polarity effect or whether it is a concomitant process. However, it would appear to be safe to assume that chemical composition, polarity, and cytoplasm are all co-ordinate factors governing the differentiation process.

At the time that increase in acidophilia is evidenced by the maturing pollen grain, the vegetative nucleus can be seen to have decreased in volume. This cannot be correlated with DNA content, and therefore suggests that the two processes may be inter-related, involving transfer of proteins to the cytoplasm. But no direct evidence can be brought to bear on this point at the present time.

In conclusion I would emphasize that studies such as those presented here do demonstrate that cytochemical techniques *can* be used to obtain

information with respect to the chemical composition of nuclei. It is possible by the use of such methods to investigate such fundamental processes as gametogenesis, growth and differentiation. It is also abundantly clear that much more work needs to be done before these processes may be completely evaluated in chemical terms.

Summary.

1. The morphology of gametogenesis in *Tradescantia paludosa* is reviewed.

2. Microscopic photometric measurements indicate (a) a progressive increase in DNA content from the time of tetrad formation until microspore mitosis; (b) there is equipartition of DNA between generative and vegetative nuclei; (c) that there is a further increase in DNA content of the vegetative nucleus prior to anthesis, although it is not possible to measure DNA content in the generative nucleus at this stage; (d) that the pollen nuclei initially contain the diploid amount of DNA in a haploid set of chromosomes; and (e) there is very good agreement between the results of the present work and microchemical findings published elsewhere by OGUR and co-workers (1951).

3. A similar cycle obtains in the case of the proteins; the rate of synthesis during development is however much faster than in the case of the DNA. The data also indicate a rapid synthesis of protein by the vegetative nucleus immediately following the microspore division. Also there appears to be a correlation between the onset of cytoplasmic basophilia and the decrease in volume of the vegetative nucleus (presumably loss of protein), just prior to anthesis.

4. Observations on acid and basic staining reactions are discussed in relation to growth and differentiation of the microspore and pollen grain.

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ANALISI CITOLOGICA DI BATTERI NELLE VARIE FASI DI ACCRESCIMENTO.

Di

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Con 3 figure nel testo.

(Eingegangen am 4. Mai/11. Juni 1951.)

Introduzione.

Con i metodi in uso fino a pochi anni or sono, lo studio morfologico di colture batteriche, in fase di accrescimento, si è limitato all'osservazione della cellula batterica in toto per quanto ne riguarda le dimensioni. Il comportamento dei batteri da questo punto di vista, fu studiato ampiamente da Henrici (1928), Huntington e Winslow (1937), Winslow e Walker (1939) mettendolo in relazione con la fase della coltura dal punto di vista dell'accrescimento numerico. Contemporaneamente, sia usando tecniche già note, sia introducendone di nuove, fu intrapreso lo studio della struttura cellulare, allo scopo di chiarire la questione della presenza nei microorganismi di un apparato nucleare assimilabile per morfologia e funzione a quello degli organismi superiori. I primi a mettere in evidenza con sicurezza un apparato nucleare vero e proprio nei batteri, o per lo meno un complesso ritenuto cromatinico, furono Stoughton (1929) valendosi della colorazione vitale con fucsina e Badian (1933) usando la colorazione bleu di metilene-eosina; ma i risultati più evidenti vennero ottenuti da Stille (1937) e Piekarski (1937), prima con la colorazione di Feulgen e poi usando l'idrolisi con acido cloridrico e la colorazione di Giemsa. La documentazione più completa sia per chiarezza, sia per ampiezza di dati illustrativi fu però offerta da Robinow (1942, 1945) con la tecnica di Piekarski da lui modificata. Come complemento allo studio dell'apparato nucleare nei batteri vanno ricordate le ricerche sul metabolismo degli acidi nucleinici nella cellula batterica (Malmgren, Thorell, Bjerklund e Caspersson 1945, Malmgren e Hedén 1947) eseguite con il microscopio a luce ultravioletta. Due rassegne assai dettagliate sull'intero argomento della citologia dei batteri sono apparse recentemente (Bisset 1950, Delaporte 1950).

Per contribuire alla dimostrazione se esistono veri cromosomi e vere divisioni mitotiche nella cellula batterica, data la varietà delle

figure morfologiche fino ad ora note, e quindi la difficoltà interpretativa di esse, abbiamo intrapreso lo studio della struttura microscopica della cromatina, durante le fasi di accrescimento di una coltura, seguendone il comportamento dalla fase iniziale di riposo fino a quella di plateau, e osservando contemporaneamente la curva di accrescimento e le modificazioni di dimensioni nei batteri componenti la coltura stessa.

Materiale e note di tecnica.

In questa ricerca abbiamo adoperato un ceppo di *Escherichia coli*, proveniente dalla collezione del Dott. Robinow, che si distingue per le sue dimensioni relativamente grandi.

La tecnica usata per allestire i preparati è stata la seguente: i prelievi furono fatti ogni 30 min. iniziando dal momento della semina, fino alla sesta ora, da una piastra di agar-brodo tenuta in termostato a 35° e seminata con 0,1 cc. di sospensione batterica contenente $5 \cdot 10^8$ batteri percc. preparata da una agar coltura di 24 ore.

La tecnica usata (comunicazione personale del Dott. C. G. Hedén dell'Inst. for Cell Research, Karolinska Institutet di Stoccolma, che vivamente ringraziamo) consiste nei seguenti punti:

1. Si ritagliano con una lancetta tanti quadratini di agar da una piastra, quanti sono i preparati che si vogliono allestire e si appoggia ciascun quadratino su un portaoggetti.

2. Si fissano i batteri in vapori di acido osmico al 2% per 5 min. mettendo una goccia di fissativo in un vetrino incavato e appoggiando il portaoggetti con la fettina di agar rivolta verso l'incavatura. Il materiale usato va sempre lavato con acqua distillata e tenuto lontano dalla polvere.

3. Dopo il fissaggio si rovesciano i portaoggetti con attaccato l'agar sui copri-oggetti e si comprime dal di sopra con una lancetta tenendo ben fermi copri e porta-oggetti. Si stacca poi il quadratino di agar cercando di non farlo scivolare.

4. Si mettono i coprioggetti in termostato a 35° per 2 min.

5. Si immergono poi per un tempo da 30 sec. a 1 min. in una soluzione di due parti di sublimato corrosivo saturo e una parte di alcool assoluto.

6. Si lasciano i coprioggetti in alcool a 70° per due ore.

7. Si asciuga in termostato a 35° per 2 min.

8. Si eseguisce l'idrolisi in HC 1 normale per 6—8 min. a 60° (avendo cura di non superare tale temperatura) e si sciacquano prima in acqua di fonte e poi in acqua distillata, due volte.

9. Per la colorazione è stato usato il *Gurrs improved Giemsa stain R. 66* in concentrazione di 2—3 gocce per cc. di tampone (fosfato sodico e potassico a p_H 7). I coprioggetti devono galleggiare nel colorante, dove rimangono due ore.

10. Per montare si immergono i coprioggetti in acetone puro per 1 min, poi con rapido passaggio in miscele di acetone e xilolo nelle seguenti proporzioni: 14:6, 6:14 e infine in xilolo puro. Indi si montano i coprioggetti sui portaoggetti usando il D.P.X. (Brit. Drug Houses) come mezzo. L'osservazione è stata eseguita con microscopio Koristka, usando una lente ad immersione ad olio A.N. 1,32 e oculare compensatore 12, con lampada e luce puntiforme e schermo giallo.

La curva di accrescimento eseguita parallelamente è stata ottenuta nel seguente modo: all'inizio si seminano con la stessa quantità di sospensione batterica diverse piastre, in modo da poter seguire la moltiplicazione dei batteri. A questo fine si lava ogni ora una piastra con 5 cc. di soluzione fisiologica (tenendo intanto quelle non ancora usate in termostato a 35°); sopra la sospensione così ottenuta si fanno i conteggi dei batteri vivi, facendo opportune diluizioni, seminando in piastra e contando il numero di colonie che si sviluppano.

Osservazioni.

Per ogni prelievo furono allestiti 4 vetrini e contati circa 250 batteri. I caratteri di ciascun prelievo sono i seguenti:

Nel primo, cioè dopo 30 min. della semina, i batteri si presentano piccoli, tozzi, di forma tondeggiante e allungata con colorazione intensa e diffusa a tutta la cellula. L'aspetto dei batteri e l'intensità di colorazione sono uniformi (fig. 1, A 1 e 2,); in alcune delle osservazioni fatte si nota la presenza di filamenti di lunghezza variabile, sottili, con andamento sinuoso, anch'essi uniformemente ed intensamente colorati (fig. 1, D 1).

Nel secondo prelievo (ore 1 dalla semina) l'aspetto dei batteri è più vario. Accanto alle forme precedentemente descritte, che si trovano immutate nel loro aspetto, ve ne sono altre che, pur avendo una colorazione diffusa a tutta l'area cellulare, presentano una strozzatura al centro più o meno evidente (fig. 1, A 3 e 4) e altre con o senza strozzatura, nelle quali è evidente una differenziazione fra sostanza citoplasmatica e cromatina, che qui appare divisa in due masse compatte ed intensamente colorate, poste ai poli delle cellule (fig. 1, B 1—4). Questi ultimi tipi hanno generalmente forma allungata e appaiono di dimensioni un poco superiori a quelli precedentemente descritti.

Sono pure presenti forme allungate senza strozzatura con 2, 3 o 4 granuli cromatinici posti perpendicolarmente all'asse maggiore del batterio o variamente inclinati. In queste cellule la colorazione è meno intensa, e la descrizione dei contorni e della forma esatta dei granuli è difficile (fig. 1, C 1—3).

È da notare la presenza di filamenti sottili e sinuosi nei quali la sostanza cromatica pur essendo diffusa presenta delle aree più colorate; è difficile dire se esse siano delle entità distinte l'una dall'altra o se si tratti di una disposizione a catena della cromatina nei filamenti, che non presentano variazioni di spessore nella loro lunghezza (fig. 1, D 2).

Nel prelievo delle ore 1,30 min. dalla semina, l'aspetto dei batteri è molto variabile, sia nello stesso preparato, sia nei vetrini allestiti durante i diversi esperimenti. Le forme nelle quali la sostanza cromatica occupa l'intera area cellulare e nelle quali essa è disposta in due masse ai poli del batterio sono rare, frequenti invece sono le forme contrassegnate col 1—3, fig. 1, C prima descritte ed altre, nelle quali la cromatina si presenta come bastoncini sottili ed avvicinati, variamente disposti nella cellula (fig. 1, C 4 e 5); gli aspetti più tipici sono quelli in cui i bastoncini hanno una forma allungata, sono piuttosto spessi e con margini uniti.

In queste cellule i bastoncini cromatici sono disposti o a coppie o sono equidistanti fra loro, presentando inclinazione diversa nella cellula (fig. 1, C 6—8).

È da notare che in alcune cellule i bastoncini descritti si presentano nitidi, di colorazione rosso-viola, mentre il citoplasma circostante è colorato in rosso pallido; in altre cellule questi stessi granuli sono circondati da un alone rosso-viola di media intensità ed altri batteri ancora presentano una colorazione di fondo rosa violacea diffusa, ed in essa si vedono masse più intensamente colorate, mal descrivibili morfologicamente (fig. 1, C 9—10). Già in questo prelievo sono presenti dei filamenti dello stesso spessore dei batteri nei quali la sostanza cromatica ha lo stesso aspetto di quello descritto precedentemente nei batteri; questi filamenti hanno un aspetto rigido e sono di lunghezza variabile, paragonabili a lunghe catene batteriche senza segni di strozzatura, che indichino una iniziale divisione (fig. 1, D 3).

In questo prelievo le dimensioni dei batteri sono superiori a quelle osservate precedentemente.

I prelievi delle ore 2,30 min., 3, 3,30 min. e 4 dalla semina possono essere illustrati insieme. Già alle ore 2, sono completamente scomparse le forme che invece prevalevano a 30 min dalla semina; l'aspetto e le dimensioni dei batteri sono molto più uniformi e alle ore 2,30 essi raggiungono le dimensioni che conserveranno fino alle ore 4 circa dalla semina.

I bastoncini cromatinici appaiono più grossi, meno compatti e con la stessa forma prima descritta; è netta la tendenza a riunirsi in coppie molto avvicinate o unite per un estremo come ben si vede in fig. 1, C 11—17. Nei conteggi abbiamo considerato come batteri le cellule contenenti fino a quattro coppie di bastoncini cromatinici, mentre quelle con numero superiore sono state classificate come catene batteriche. Va notata pure la presenza di cellule nelle quali i bastoncini, anzichè avere i margini dritti, presentano delle fini ondulazioni, una forma meno regolare e varia intensità di colorazione (fig. 1, C 17—20). I bastoncini ora descritti hanno talora un alone rosa violaceo di media intensità, come i batteri di cui si è detto sopra (fig. 1, C 21). Queste cellule in genere hanno dimensioni superiori alle altre, sopra tutto per quanto riguarda lo spessore. Rarissima la presenza di cellule del tipo 22, fig. 1, D nelle quali la massa cromatica è raccolta al centro della cellula, con l'aspetto di un bastoncino lievemente ondulato. I filamenti assai numerosi hanno l'aspetto di lunghe catene batteriche, e in esso la differenziazione tra sostanza cromatinica e citoplasma è ben netta.

Alle ore 4,30 dalla semina i batteri hanno un aspetto molto variabile. Accanto alle forme ben differenziate, se ne notano altre nelle quali i bastoncini cromatinici sono circondati dall'alone rosa-violaceo precedentemente descritto, e altre in cui la sostanza cromatica è ormai compatta e disposta ai due poli della cellula; però non si osservano forme nuove rispetto a quelle precedentemente illustrate. I batteri hanno

dimensioni un poco più piccole e sono sempre presenti dei filamenti variamente differenziati.

Dall'ora 4,30 fino alla sesta ora, l'aspetto dei batteri è simile a quello già descritto nei prelievi delle ore 1 e 30 min., cioè le forme differenziate

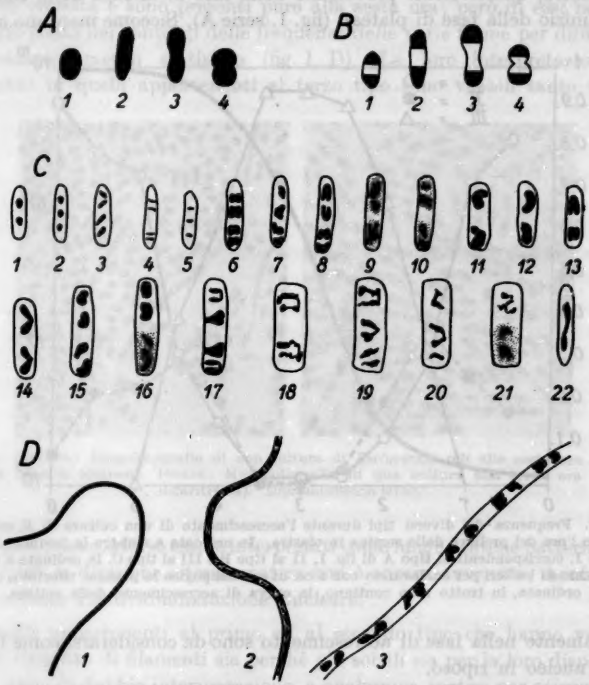

Fig. 1. Aspetti morfologici di *Escherichia coli* raggruppati secondo le varie forme. A Forme in riposo. B Forme di passaggio. C Forme in accrescimento. D Filamenti.

diminuiscono di numero, mentre dapprima prevalgono quelle con due masse cromatiniche disposte ai poli della cellula, che lasciano poi il posto alle cellule completamente indifferenziate. La modalità di passaggio da uno stato all'altro è sempre la stessa, però i batteri si conservano di volume un poco superiore a quello iniziale. I filamenti sono sempre presenti e sono numericamente superiori a quelli visti al principio della coltura. Tutte le varie forme di batteri, osservate nei prelievi eseguiti e ripetutamente controllati, furono distinte in tre tipi.

Nella fig. 2 sono riportate le frequenze rispettive dei tre tipi di cellule, in relazione alle varie fasi di accrescimento della coltura.

I° gruppo — Forme indifferenziate: nelle quali la sostanza cromatica appare diffusa a tutta la cellula batterica; come precedentemente si è detto, queste forme prevalgono nella fase di latenza della coltura e all'inizio della fase di plateau (fig. 1, serie A), Siccome mancano com-

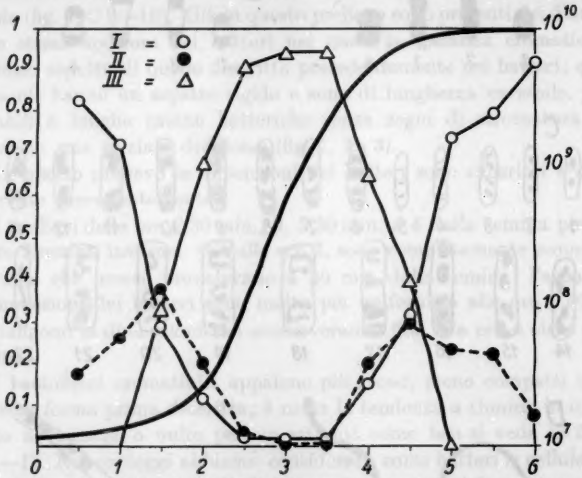

Fig. 2. Frequenza dei diversi tipi durante l'accrescimento di una cultura di *E. coli*. In ascissa l'ora del prelievo dalla semina in piastra. In ordinata a sinistra la frequenza delle forme I, corrispondenti al tipo A di fig. 1, II al tipo B e III al tipo C. In ordinata a destra il numero di batteri per cc. lavando con 5 cc. di sol. fisiologica la piastra; riferita a questa ordinata, in tratto nero continuo, la curva di accrescimento della coltura.

pletamente nella fase di accrescimento sono da considerarsi come forme con nucleo in riposo.

II° gruppo — Forme di passaggio: nelle quali la sostanza cromatica appare divisa in due masse ai poli della cellula; le forme appartenenti a questo gruppo compaiono in corrispondenza di due punti della curva di accrescimento: verso la fine della fase di latenza e al principio della fase logaritmica e poi alla fine di quest'ultima e durante l'iniziarsi della fase di plateau (fig. 1, B). Rappresentano quindi uno stato di differenziazione incompleta e di passaggio.

III° gruppo — Forme differenziate: nelle quali la cromatina appare con gli aspetti precedentemente illustrati; la varietà dei tipi raccolti in questa categoria è notevole, e probabilmente essi possono avere un significato particolare nel processo della divisione; però in base alle osservazioni fatte si è ritenuto opportuno non tenerli distinti, e con-

siderarli tutti come forme di moltiplicazione dato che compaiono solo durante la fase logaritmica della curva di accrescimento (fig. 1, C).

Furono presi in considerazione anche i filamenti; essi sono assai rari all'inizio della fase di latenza, aumentano di numero verso la seconda ora di crescita e sono presenti pure alla sesta ora; però di essi non fu tenuto conto nei conteggi delle frequenze delle varie forme per difficoltà di campionamento statistico (fig. 1, D). La loro interpretazione è incerta: in quelli appartenenti al terzo tipo sono visibili tante unità

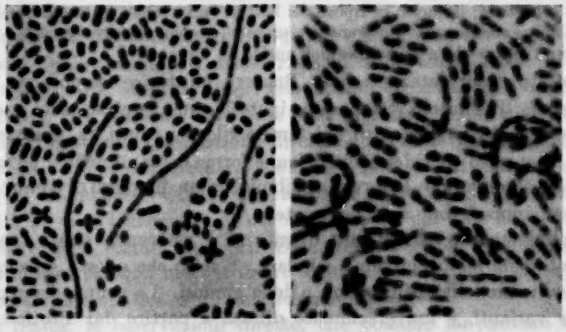

Fig. 3. Sinistra: Microfotografia di una coltura di *Escherichia coli* alla sesta ora dalla semina (fase di plateau). Destra: Microfotografia di una coltura alla terza ora (fase logaritmica). Ingrandimento 2750.

cellulari e possono forse essere interpretati come lunghe catene batteriche, nelle quali la divisione citoplasmatica non è ancora avvenuta, mentre è già evidente l'individualizzazione nucleare.

Quelli appartenenti al primo ed al secondo tipo che hanno veramente l'aspetto di filamenti sia perchè più sottili sia per la loro disposizione, sono di dubbia interpretazione, e qualunque ipotesi per spiegarne il significato sarebbe azzardata.

Discussione.

I dati osservati ci permettono di portare un contributo alla affermazione che i corpi cromatici osservati sono da ritenersi cromosomi, data la loro comparsa nella fase di accrescimento e la costanza del loro aspetto confermata dalle osservazioni di autori precedenti (Robinow, Bisset); inoltre il numero dei bastoni cromatici nella cellula appare sempre il medesimo e la loro tendenza a disporsi a coppie richiama il comportamento dei cromosomi nella anafase mitotica degli altri organismi viventi.

Il problema delle *modalità di divisione* della cromatina è piuttosto complesso. Il Bisset è l'unico autore che fino ad ora abbia avanzato uno schema del modo di riproduzione dei batteri; in quelli non sporigeni essa avverrebbe nel modo seguente:

In colture di poche ore di età i cromosomi in numero di quattro per cellula si fissurerebbero trasversalmente, successivamente comparirebbero delle forme cosiddette di fusione con materiale cromatico più o meno ben differenziabile in elementi distinti, da cui deriverebbero dei filamenti costituiti da tante unità batteriche avvicinate e senza membrana interposta, che a loro volta si dividerebbero per dare origine o alle forme a nucleo cosiddetto secondario o a quelle con nucleo a bastone; il nucleo in riposo dei batteri deriverebbe da una di queste due ultime forme considerate. Un eventuale processo di coniugazione sessuata potrebbe ntervenire prima della comparsa del nucleo in riposo.

Questa interpretazione del Bisset, piuttosto complessa, viene presentata dall'autore stesso come ipotetica in alcuni dei suoi passaggi. Pertanto nelle condizioni in cui furono rilevati i dati da noi osservati e per il ceppo in considerazione, alcune forme come: il nucleo di fusione, il nucleo secondario e le forme attribuibili a un processo di coniugazione sessuata, non furono mai osservate.

Dobbiamo ora paragonare quanto si è osservato con i fenomeni generali della moltiplicazione cellulare. È noto che questa è conosciuta con le due modalità della *mitosi* e della *amitosi*. La prima, sia pure con alcune varianti, è universalmente diffusa a partire dai Protisti, ed è caratterizzata dalla divisione longitudinale dei cromosomi, i cui cromatidi si separano mediante il fuso, la seconda, assai rara e forse di dubbia esistenza all'infuori che nei macronuclei dei Ciliati, consta di una ripartizione della cromatina nei nuclei figli, senza che compaiano veri cromosomi e senza che si formi il fuso.

La riproduzione del batterio studiato non entra a rigore nel primo tipo per la mancanza del fuso. La stessa divisione longitudinale dei corpi cromatici è più inferita per ragioni di struttura del genoma (Lederberg 1946, 1947; Cavalli e Heslot 1949) che per osservazioni dirette. Gli argomenti più probanti si ricavano dalle posizioni parallele dei bastoncini cromatici nella fig. 1., C che tuttavia non sono facilmente interpretabili come anafasi.

D'altra parte non si può comprendere tutta la varietà dei fenomeni descritti nella definizione di amitosi. Infatti, le moltiplicazioni rappresentate nella fig. 1, B possono essere definite amitotiche; ma le più complesse forme della fig. 1, C mostrano corpi che appaiono definiti per struttura e per numero, in modo da far pensare direttamente a cromosomi che sarebbero due o quattro; per tacere dei filamenti a catena. Siccome queste forme sono tipiche della fase logaritmica o di accrescimento, è logico concludere che i corpi sono effettivamente cromosomi o aggregati di essi, i quali nell'amitosi non sogliono comparire.

Si può dunque affermare che in una popolazione di *E. coli* in accrescimento si riscontrano divisioni amitotiche e divisioni pseudomitotiche senza fuso apparente, in punti tipici della curva. Le forme pseudomitotiche allo stato attuale delle nostre conoscenze, sembrano stare in un gruppo a sè fra la mitosi e la amitosi. Il fatto che le forme cosiddette indifferenziate o amitotiche e le differenziate o pseudomitotiche si riscontrino in momenti caratteristici della curva di accrescimento fa ritenere che la loro comparsa sia controllata dalle condizioni fisiologiche della cellula. Ciò può tuttavia essere espresso solo in forma ipotetica e vaga, allo stato attuale delle ricerche.

Il *nucleo in riposo* dei batteri in genere e particolarmente nella forma studiata, appare come una massa ricca di acido timonucleinico, senza struttura interna. Fra gli autori che si sono occupati della cromatina nei batteri, due punti di vista sono stati espressi. Quello di Robinow e di altri, legati alla osservazione diretta, affermante l'esistenza di una masserella compatta di cromatina, e quello, più ricco di elementi speculativi, di Caspersson (1947) che tenta di riportare la struttura del batterio in quella generale, di cui sono esempi più noti le cellule dei Metazoi e delle piante superiori. Secondo questo punto di vista non si vedrebbero ostacoli nell'attribuire al nucleo batterico la presenza di una parte eucromatica e di una eterocromatica.

In realtà, le osservazioni dirette, che possono far trovare questi elementi strutturali anche nei lieviti (Lindegren 1946; Caspersson 1947) non soccorrono per i batteri, ove ogni affermazione conserva fino ad oggi un puro valore ipotetico.

Le nostre osservazioni non concedono di portare nuova luce sul problema delle parti differenziate del nucleo batterico in riposo. Possiamo invece discutere brevemente i seguenti punti:

1. il nucleo in riposo non si differenzia dal citoplasma delle forme indifferenziate (fig. 1, A).

2. esso appare costituito da corpi cromatici isolati, non inclusi in una membrana nucleare unica nelle forme differenziate (fig. 1, C) dove però è difficile dire quando si possa parlare di un vero riposo nucleare. Questo può essere più logicamente ammesso per le rarissime forme come quelle rappresentate al numero 22 della fig. 1, C.

3. analogo aspetto di masse compatte (cromosomi) isolate nel citoplasma senza inclusione in un'unica membrana, si riscontra nei filamenti.

Il primo punto presenta il problema di come interpretare una cellula senza differenziazione fra nucleo e citoplasma. Se vogliamo seguire la tendenza consistente nel voler stabilire analogie tra la cellula batterica e quella degli organismi superiori, dobbiamo escludere che nucleo e citoplasma non sono differenziati, ma piuttosto che, come nei linfociti,

la massa citoplasmatica sia irrilevante in confronto a quella del nucleo; se invece si segue il concetto che nessuna necessità impone di trovare analogie fra batteri ed altri organismi ogni ipotesi può essere lecita, ma è prematuro e inutile quindi insistere oggi su ciò.

Il secondo punto ci pone il problema di paragonare il nucleo batterico con quello degli altri organismi, anche di struttura appena superiore, ciò che colpisce è l'assenza di membrana nucleare o di nucleoli, onde si può dire che la cromatina differenziata non forma un nucleo, ma solo un sistema di corpi cromatici compatti, di aspetto simile a quello dei cromosomi mitotici. Paragoni con altri gruppi di organismi non sono possibili; infatti i soli che posseggono un tipo di cromatina a masse isolate e compatte sono i Ciliati (Devidé e Geitler 1947; Barigozzi 1949). Ma i macronuclei di questi non sono probabilmente paragonabili se non per considerazioni puramente morfologiche ed esteriori. Si può concludere che la cromatina nei batteri è o indifferenziata o differenziata in bastoncini di numero praticamente constante e variabile in serie multiple, cui può competere il nome di cromosomi, che permangono isolati nel citoplasma nella fase logaritmica. Forse è meno legittimo parlare di un vero nucleo trattandosi di un sistema di corpi isolati. Il termine di „nucleo in miniatura" (Jordan 1939), così spesso usato, sembra dover cadere perchè, riferito alla massa del batterio, il complesso delle parti di cromatina non è affatto piccolo. Si può prospettare in forma provvisoria che il genoma dei batteri sia legato ai corpi cromatinici, che per la loro differenziazione nei fenomeni riproduttivi, meritano il nome di cromosomi. Circa il significato delle lunghe catene filamentose, per ora preferiamo di non aggiungere una disamina particolare.

Zusammenfassung.

Es wird das Problem des Chromatins von *E. coli* an Hand von Robinows Technik untersucht. Der Vergleich zwischen den Färbungsbildern von bestimmten Stufen des Wachstums zeigt, daß regelmäßig geformte Chromatinmassen nur zustande kommen, wenn die Bakterien Vermehrungsvorgänge durchmachen. Dieses Verhalten wird als pseudomitotisch interpretiert, was wahrscheinlich macht, daß die Chromatinmassen mit Chromosomen zu vergleichen sind.

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THE CYTOLOGY AND CHEMICAL NATURE OF SOME CONSTITUENTS OF THE DEVELOPING SPERM.

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With 18 figures in the text.

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Contents.

I. Introduction.

The acrosome or apical element as well as other cytoplasmic constituents of the animal spermatozoon have long been of interest to biologists. The careful descriptions of the mature sperms of many species of animals published by BALLOWITZ and RETZIUS about half a century ago established the general features of the sperm and showed that the acrosome is subject to much variation in shape and position. In 1917, GATENBY discovered that in the formation of the acrosome the Golgi material plays a role and that relationship was the subject of much study in later investigations. Perhaps the most exact and detailed of such investigations were those of BOWEN who followed the manoeuvres of the Golgi bodies or dictyosomes from the spermatogonia, through the spermatocytes, to the sperm and showed that they are indeed intimately involved in the formation of the acrosome. It was BOWEN ('24) who also, like LOEB and LILLIE before him, suggested that the acrosome might play an important role in the process of fertilization—a role perhaps akin to that of an activator or enzyme that initiates the first steps in the development of the egg.

Although more recent work (see for instance HIRSCH '39 and WORLEY '46) has shown that the intricate, net-like conformation so often assumed by the Golgi material in certain somatic cells probably rests on the effects of fixation, such is not the case in the male germ cells. The platelets or dictyosomes which there represent the Golgi material are easily visible in living cells subjected to a vital dye such as methyl blue (WORLEY '39) and, indeed, in untreated normal cells as well (JOHNSON '31). In such material the dictyosomes differ little in conformation from what is seen after careful fixation and there is thus no reason to question descriptive work like that of BOWEN on fixed germ cells.

The present investigation is concerned with the relations between the dictyosomes and the acrosome, as well as with certain aspects of the mitochondrial Nebenkern. In our cytological as well as cytochemical analysis we have benefited by the use of the method of MCMANUS ('46), the principles of which were arrived at independently by HOTCHKISS ('48). It is the latter's technique which proved especially advantageous to us.

As in a previous study (SCHRADER and LEUCHTENBERGER, '50), we utilized in our analysis the advantageous conditions presented in the testis of the hemipteran insect, *Arvelius albopunctatus*. In every male of this species there are three different size classes of spermatocytes and spermatids, and each of these classes is confined to certain sections or lobes of the testis. In the fourth lobe only small spermatocytes and spermatids are present; the first, second and sixth lobes have cells of the common or normal size; while in the third and fifth lobes there are very large cells. The chromosome size and amounts of desoxyribose nucleic acid (DNA) are identical for any given stage in all meiotic nuclei of the testis. However, this constancy of the DNA is not correlated with a corresponding constancy in other cell constituents. Thus the cells of the large-cell lobes show not only a definite growth as indicated by the quantity of proteins in the extra-chromosomal substance of the nucleus, but also a proportionate increase in the ribose nucleic acid (RNA) and proteins of the cytoplasm. In short, there are definite quantitative differences in certain synthetic activities of the three sizes of closely related cells, and these differences offer certain advantages in analyzing various cytological features of the cell—in the present case, the Golgi bodies or dictyosomes and the acrosome of the sperm on the one hand, and the mitochondrial Nebenkern on the other. The size differences that obtain, for instance in the acrosomal mass, in adjoining lobes of the testis are shown in the photographs (Figures 1 and 2).

Certain of our findings were reported in a preliminary publication (LEUCHTENBERGER and SCHRADER, '50). The present paper brings a

more detailed consideration of those findings as well as further steps in the analysis.

II. Material and Methods.

Male specimes of *Arvelius albopunctatus* were collected in Costa Rica during April and May in 1950. The testes were fixed in Carnoy (1 acetic acid; 3 absolute alcohol), and imbedded and sectioned at various thicknesses, according to the requirements of the investigation. The photometric microscopic measurements were made by the method originally described by POLLISTER and RIS ('47). A description of the apparatus in its present form and a discussion of the photometric microscopic method are given by LEUCHTENBERGER ('50) and SWIFT ('50).

For the identification of the polysaccharides in the cell structures, the microchemical periodic acid Schiff (PAS) reaction as described by HOTCHKISS ('48) and MCMANUS ('46) was used. According to these authors the reaction of the periodic acid with carbohydrates when present as 1,2 glycol grouping is considered to be the following:

$$\begin{array}{l} \text{OH OH} \\ \text{R—C—C—R} + \text{HIO}_4 \rightarrow 2\,\text{R—CHO}. \end{array}$$

The aldehydes which are formed after periodic acid oxidation from 1,2 glycols in sections form a colored complex with the Schiff reagent. Control sections, without periodic acid oxidation, did not show any development of color after exposure to the Schiff reagent.

In order to characterize the types of polysaccharides in our tissues we used the PAS reaction in combination with the acetylation technique of MCMANUS and CASON ('50) and with enzymes such as amylase, diastase, pepsin, trypsin and various hyaluronidases (derived from bull testis and bacteria). Concentration of enzymes and time of exposure of sections are given in Table 2.

The amount of polysaccharides was estimated on the basis of the intensity of the Schiff color after periodic acid oxidation in individual cells, from photometric microscopic measurements of the absorption of the 546-millimicrons line from a Mercury lamp isolated by a Wratten 62 filter.

For the absorption measurements of the Schiff color in the sections, acrosomal material of spherical shape was selected in large and in normal-sized cells which were in the same stage of development and were present on the same slide. Photometric measurements of the acrosome in the small cells were not possible due to their small dimensions. The amounts of polysaccharides are expressed in arbitrary units (PAS units) and are obtained by multiplying the extinctions by the square of the radius of the sphere of the acrosome.

For the determination of proteins in the acrosome and Nebenkern the Millon reaction was used and the color measured photometrically as previously described.

An estimation of the basic proteins in the Nebenkern and acrosome was attempted by the use of Fast green as previously described by SCHRADER and

Fig. 1. 6th (left) and 5th (right) lobes in testis of *Arvelius*. The dark, more or less spherical masses in each represent the PAS-positive, acrosomal masses, prior to elongation. In the 6th lobe they are about 1/40 as large as in the 5th lobe. PAS counter-stained with methyl green. Photo 320 ×.

Fig. 2. 6th (upper) and 5th (lower) lobes in region closer to sperm duct than that of Fig. 1. Sperm bundles well formed in 6th lobe. The much greater dimensions of the acrosomes in the 5th lobe is shown in the partially cut bundle seen there. (Sperm bundles indicated by arrows.) PAS counter-stained with methyl green. Photo 320 ×.

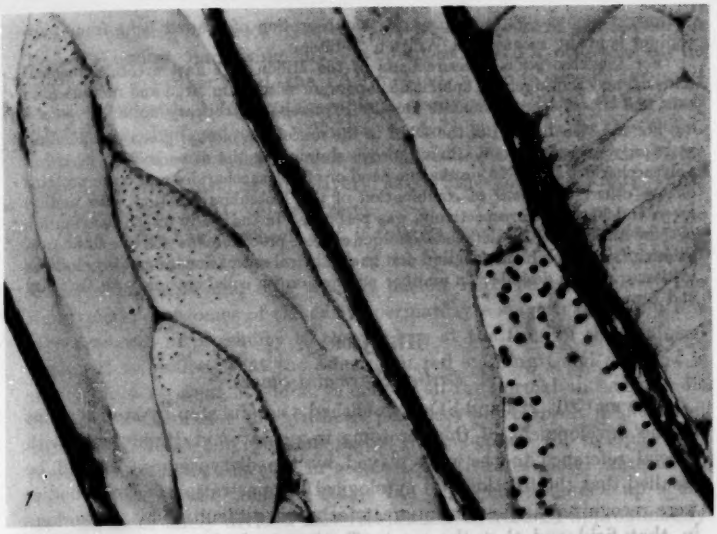


Fig. 1.

Fig. 2.

LEUCHTENBERGER ('50) and measured as absorption of the red light from a zirconium lamp by means of a Wratten 26 filter.

For the absorption measurements of the Millon and Fast green, acrosomal material and Nebenkern of spherical shape were selected in large and normal-sized cells and the measured amounts are also expressed in arbitrary units.

For the visualization of the DNA in the nuclei the methyl green and Feulgen reaction were carried out. Simultaneous staining in the same section of either polysaccharides and DNA on the one hand or of polysaccharides and basic proteins on the other, by means of combination of the Hotchkiss reaction with methyl green and Fast green respectively, was performed in the study of the distribution of these substances. The detailed technique of these procedures as well as a standardization of the Hotchkiss reaction for quantitative estimation of polysaccharides in tissues will be described in another publication (in collaboration with ORBISON and LIEB).

III. Cytology.

A. Earlier Spermatid Stages.

BOWEN ('20, '22a and b) has published a careful step-by-step account of the development of the acrosome in pentatomid Hemiptera, with special reference to the part played by the dictyosomes. It will be recalled that the fixation and cytological demonstration of Golgi bodies were recognized as presenting great technical difficulties by all workers in that field and that the most effective methods, such as those of Kopsch, Weigl and Kolatchev, involved fixing fluids without acetic and a later, prolonged treatment with osmic acid (OsO_4 in aqueous solution). In preparations made by such methods, the dictyosome appeared as a platelet with a darkly staining shell or periphery and a more lightly colored interior (see general review by HIRSCH, '39). The shell (HIRSCH's Golgi-externum) is by most workers considered to be composed of lipoids (phosphatides and unsaturated fatty acids) and this explains why the Golgi-externum is blackened by OsO_4 and why the presence of acetic acid or fat solvents in the fixing fluid makes good preservation of Golgi bodies impossible. The nature of the more fluid contents (WORLEY, '46) is more uncertain. According to TENNENT, GARDINER and SMITH ('31), methods such as those of Kopsch and Kolatchev distinguish only between the presence and absence of unsaturated fatty acids and hence the light stain of the Golgi-internum probably indicates non-lipoid contents.

This shows that it was recognized by some of the earlier workers that although the generally accepted Golgi techniques made it possible to trace the manoeuvres of the dictyosomes, these methods might nevertheless fail to preserve all of the dictyosomal constituents. The present investigation with a new technique supports this view. Our preparations do not show the darkly stained periphery of the dictyosome which is so characteristic after the older treatments and that

is what one might expect from the destructive effects of acetic acid on lipoids (Carnoy, our fixing fluid, is composed of 25% of glacial acetic acid). On the other hand, the PAS method, which is specific for polysaccharides with 1,2 glycol grouping, stains what is left, after such fixation, a vivid red color. This is to be identified with the Golgi-internum of the dictyosome and through it the behavior of the latter can be traced just as effectively as by the older methods which primarily stain the Golgi-externum.

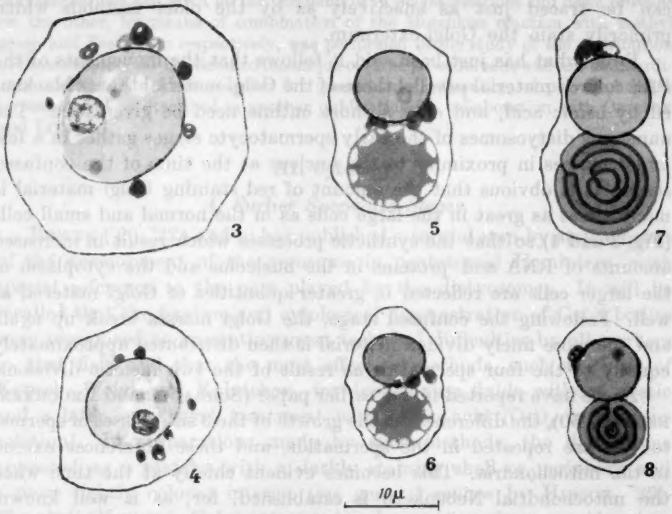
From what has just been said it follows that the movements of the PAS colored material parallel those of the Golgi material that is blackened by osmic acid, and only a short outline need be given here. The numerous dictyosomes of the early spermatocyte stages gather in a few larger masses in proximity to the nucleus at the time of the confused stage. It is obvious that the amount of red staining Golgi material is many times as great in the large cells as in the normal and small cells (Fig. 3 and 4) so that the synthetic processes which result in increased amounts of RNA and proteins in the nucleolus and the cytoplasm of the larger cells are reflected in greater quantities of Golgi material as well. Following the confused stage, the Golgi masses break up again and the more finely divided material is then distributed approximately equally to the four spermatids as result of the two meiotic divisions.

As we have reported in our earlier paper (SCHRADER and LEUCHTENBERGER, '50), the differences in the growth of the 3 size classes of spermatocytes are repeated in the spermatids, and these differences extend to the mitochondria. This becomes evident chiefly at the time when the mitochondrial Nebenkern is established, for, as is well known, during the period at which the so called "onion pattern" is laid down, the Nebenkern is peculiarly resistent to acids and appears even after Carnoy fixation (Figs. 5–8). After a strong acid stain like Fast green the features of the Nebenkern can then be made out and there can be no doubt that the Nebenkern is considerably more voluminous in the large cells than in the two smaller size classes.

It is at this time too that the red, dictyosomal bodies once more come together in larger masses which finally gather in a single mass in contact with the nucleus (Fig. 9). This mass or body of course undergoes the same movements as the osmic acid blackened "acroblast", that BOWEN ('22a) has described. Keeping in close proximity to the nuclear wall there is a shift to the region close to the Nebenkern and then a movement toward the opposite side of the nucleus which represents the more anterior region of the future sperm. Usually it comes to rest at one side of the nucleus and further development usually takes place there. It was not possible to follow the differentiation of this mass or acroblast into BOWEN's acrosomal body and Golgi remnant.

Possibly the Golgi remnant, which is discarded and sloughed off into the tail, is not stained by the PAS method, but a closer study is necessary to establish that fact.

There is little question that the red mass which remains is directly involved in the formation of the acrosome and may well be called acrosomal material. All through these early stages of the spermatid



Figs. 3—8. Fig. 3. First spermatocyte confused stage in large cell of 5th lobe. Dictyosomal masses red; chromatin green; nucleolus uncolored. PAS counter-stained with methyl green. Fig. 4. Same stage as in Fig. 3, but in normal-sized cell of 2nd lobe. PAS counter-stained with methyl green. Fig. 5. Spermatid in large cell lobe. Gathering of dictyosomal masses in region between nucleus and mitochondrial Nebenkern. Latter showing first structural differentiation. PAS counter-stained with Fast green. Fig. 6. Same stage as Fig. 5, but in normal-sized cell. PAS and Fast green. Fig. 7. Spermatid in large-cell lobe. Dictyosomal material gathered in a single mass which is in migration around the nucleus to the anterior. Typical onion pattern in Nebenkern. PAS and Fast green. Fig. 8. Same stage as Fig. 7, but in normal-sized cell. PAS and Fast green.

this mass increases in volume and gains also in the intensity with which it stains by the PAS method though some evidence of vacuolization is present until a late phase of spermateleosis. At the time that it assumes a position close to the posteriorly located Nebenkern (which is now dividing and elongating) it becomes smooth in outline and is applied closely to the spherical nucleus like a small but thick cap (Fig. 10). Leaving this position for a more or less lateral one, it appears to become more liquid and seems to flow out over the surface of the nucleus until the latter is almost half covered (Fig. 11). It is only

now that the round nucleus begins to show the first indications of the elongation that is to terminate in the attenuated head of the finished sperm. The closely applied acrosomal material elongates with the nucleus (Fig. 12) but, as the succeding phases indicate, the final shaping of the acrosome cannot be an entirely passive process for it finally extends beyond the nucleus and undergoes additional transformations that are independent of the nuclear shape. Thus, a circumscribed, dark red region which is to form the very apex or perforatorium of the finished sperm becomes spear shaped and extends anteriorily beyond the elongating nucleus. This stage corresponds roughly to that of the normal sized spermatid shown in Fig. 13.

As the sperm head initiates the extreme elongation that follows the stage shown in Fig. 14, loosely coiled fibrillae make their appearance in the large acrosomes. These are PAS-positive in their staining reaction and at least six or seven of such fibrillae can be counted in each acrosome (Fig. 15). In the acrosomes of normal and small spermatids no such fibrillar structure could be distinguished with certainty, but this is perhaps attributable to the much smaller dimensions of the various spermatid structures that are present there. It is not likely that these fibrillae correspond to the skeletal fibers of KOLTZOFF ('09) for they are clearly more numerous; nor are they identical with his mitochondrial bands that wind about the exterior of the sperm head. Their smaller diameter and positive PAS reaction speak conclusively against such an identification and further analysis is evidently called for.

The cytological relationship between the Golgi material and the acrosome has also been demonstrated by LEBLOND ('50) in the rat sperm, by a similar use of the PAS method. Although the rat has no such large spermatocytes as *Arvelius* in which the various steps are seen in an exaggerated form, LEBLOND's account, albeit very brief, leaves little doubt that in general the same steps are followed.

To sum up, there is direct evidence that a polysaccharide with a 1,2 glycol linkage, which stains red in the PAS treatment, is present in the dictyosomes or Golgi bodies of the primary and secondary spermatocytes. These dictyosomes are assembled to form the acroblast in the spermatid, and the acrosome that is finally evolved is also PAS-positive in its color reaction. In the formation of the acrosome there is thus a direct utilization of the dictyosomal material that is present at least two cell generations earlier. There is, however, a considerable increase in the amount of this PAS-positive material in the spermatid.

As already mentioned, the mitochondria of the early spermatid gather in a spherical mass or Nebenkern. Somewhat later, the material of this is rearranged to form the so-called onion pattern, (Figs. 3–6), a compound structure of concentric layers which in some respects bears

a resemblance to the "corpora amylacea" that are formed at times in certain mammalian tissues. But whether this implies more than a superficial similarity can only be decided by further work.

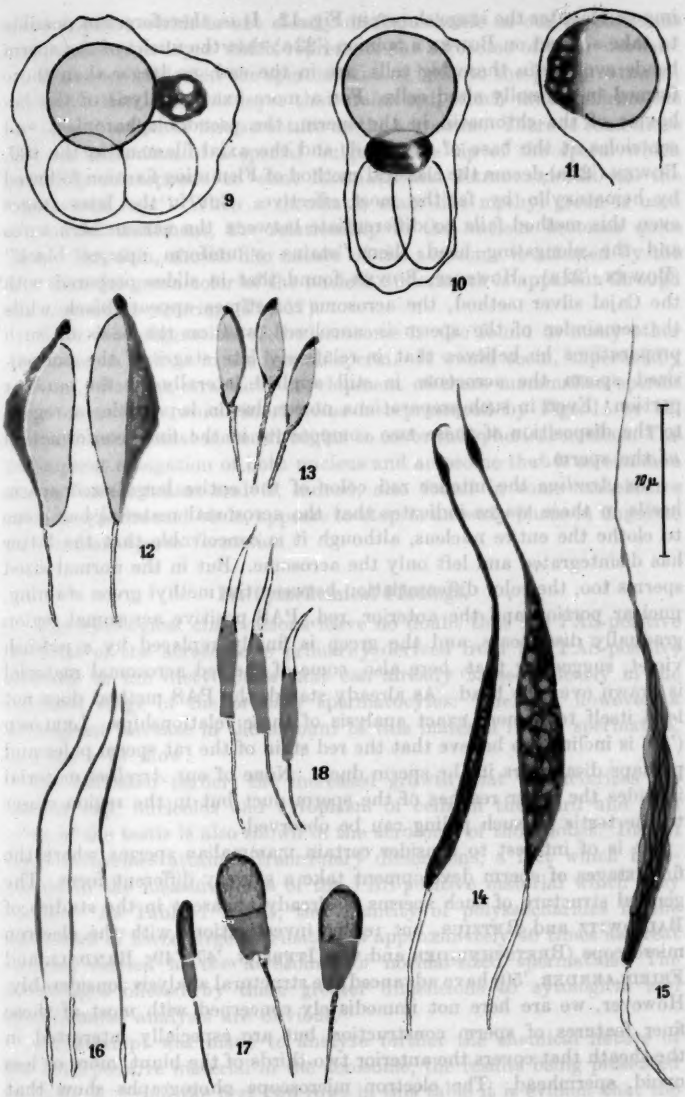
The succeeding division of the Nebenkern into two smaller spheres (Fig. 10) takes place long before there is any elongation of the nucleus. The two smaller Nebenkern spheres then lengthen and spiral around the growing tail filament of the sperm and it is during this process that the nucleus begins to attenuate. Although our treatment of the material is excellent for the chemical questions at issue, it offers no advantages in studying the later history of the Nebenkern. In view of the careful work that has already been published, we made no special effort to elucidate the detailed morphology of the mitochondrial derivatives in these final stages.

However, since in its size and shape, the earlier spherical Nebenkern offers special advantages for cytochemical and photometric determinations, and since it is clearly a derivative of mitochondrial elements, we utilized it in a comparison with the acrosome which is a Golgi body derivative. It may be remarked that the Nebenkern, like every other non-chromosomal structure, is greatly increased in size in the spermatids of the third and fifth lobes, albeit it does not reach the exaggerated dimensions which there characterize the acrosome. But in the size increase of the Nebenkern we are dealing with a true growth, just as is the case with all the other non-chromosomal structures of these lobes (Table 4).

B. Later Spermatid Stages.

As stated earlier, the PAS technique stains only the acrosomal material. After counter staining with methyl green the chromatin in the sperm head of all except the final stages is clearly defined in spermatids of the small and normal cell lobes (Fig. 13 and 16), but the great dilution of DNA in the large cells makes identification of the nucleus

Figs. 9—18. Fig. 9. Acrosomal mass in posterior migration around nucleus of large-sized spermatid. Enlarging nucleus stained more faintly than normal sized nuclei. Divided Nebenkern unstained. PAS and methyl green. Fig. 10. Cap-like form of acrosomal mass applied to posterior region of nucleus. Nebenkern halves elongating. PAS and methyl green. Fig. 11. Acrosomal mass in lateral position, spreading over surface of still spherical nucleus. PAS and methyl green. Fig. 12. Elongation of both acrosomal mass and nucleus. Darker anterior region in acro-somal mass represents future perforatorium. Elongating tail gray or shadowy (the periaxial plasma sheath does not show up in reproduction). PAS and methyl green. Fig. 13. Elongation of acrosomal mass and nucleus in normal-sized spermatid. Nucleus stains more intensely than in large sized spermatid because of greater concentration of DNA. PAS and methyl green. Fig. 14. Further elongation of acrosome in large-sized spermatid. Perforatorium more distinct. Nucleus no longer distinguishable. PAS and methyl green. Fig. 15. Elongating acrosome of large-sized spermatid, showing fibrillar structure. PAS and methyl green. (Tails in Figs. 14 and 15 should be somewhat broader than reproduced.) Fig. 16. Elongating, normal-sized spermatid in which the green nuclear portion is still clearly distinguishable. PAS and methyl green. Fig. 17. Bull sperms. PAS and methyl green. Fig. 18. Snake sperms. Showing small amount of PAS-positive material at tip of acrosome, or else none at all. PAS and methyl green.



Figs. 9—18.

impossible after the stage shown in Fig. 12. It is, therefore, not possible to take a stand on BOWEN's opinion ('22a) that the nuclei of the sperm heads evolved in these big cells, are in the end, no larger than those formed in normally sized cells. For a more exact analysis of the behavior of the chromatin in the sperm, the pseudoblepharoplast and centrioles at the base of the head, and the axial filament in the tail, BOWEN ('22a) deems the classical method of Flemming fixation followed by hematoxylin by far the most effective. But in the later stages even this method fails to differentiate between the various structures and the elongating head then "stains a uniform opaque black" (BOWEN, '22a). However, BOWEN found that in slides prepared with the Cajal silver method, the acrosome sometimes appears black while the remainder of the sperm is uncolored, and on the basis of such preparations he believes that in relatively late stages of the normal-sized sperm the acrosome is still applied laterally to the nuclear portion. Even in such preparations no conclusion is possible in regard to the disposition of these two components in the final conformation of the sperm.

In *Arvelius* the intense red color of the entire large sized sperm heads in these stages indicates that the acrosomal material has come to clothe the entire nucleus, although it is conceivable that the latter has disintegrated and left only the acrosome. But in the normal sized sperms too, the color differentiation between the methyl green staining, nuclear portion and the anterior, red, PAS positive acrosomal region gradually disappears, and the green is finally replaced by a reddish violet, suggesting that, here also, some of the red acrosomal material is drawn over the head. As already stated, the PAS method does not lend itself to a more exact analysis of these relationships. LEBLOND ('50) is inclined to believe that the red stain of the rat sperm pales and perhaps disappears in the sperm ducts. None of our *Arvelius* material includes the lower reaches of the sperm duct but in the region closer to the testis no such paling can be observed.

It is of interest to consider certain mammalian sperms where the final stages of sperm development take a slightly different form. The general structure of such sperms is already apparent in the studies of BALLOWITZ and RETZIUS, but recent investigations with the electron microscope (BRETSCHNEIDER and VAN ITERSON, '47, '49; RANDALL and FRIEDLAENDER, '50) have advanced the structural analysis considerably. However, we are here not immediately concerned with most of these finer features of sperm construction but are especially interested in the sheath that covers the anterior two-thirds of the blunt, more or less ovoid, spermhead. The electron microscope photographs show that this is itself a complex structure, composed of an outermost layer (the

galea capitis) which is not clearly distinguishable in our preparations and, under it, a layer which represents the acrosome of older workers.

When we subjected the spermatozoa of bulls to the PAS treatment, we found that it is this layer that stains red, which thus confirms the old identification of this structure as the acrosome. There is sometimes also an indication of a special bulge at the tip of the sperm which probably corresponds to what RANDALL and FRIEDLAENDER call the "acrosomal cap." When a chromatin stain like methyl green is used after PAS treatment, the relationship to the nucleus becomes quite clear. The thin, sheath-like nature of the acrosome is attested by the fact that the green color of the nucleus underneath is apparent through the acrosomal covering (Fig. 17).

Such a configuration of the acrosome is also found in many other ungulates as well as in the human sperm. It would seem, superficially at least, that the structural development of these mammalian sperms stops at a stage which in *Arvelius* is represented by Figs. 11 or 12, where the acrosomal material begins to cover the spherical nucleus. The subsequent elongation of both nucleus and acrosome that is so common among invertebrates and is, indeed, also found in some vertebrates such as reptiles and birds, appears to stop in an early phase in ungulate and human sperm formation.

IV. Cytochemical Findings.

The cytological observations leave no doubt that the PAS-positive material of the acrosome is primarily derived from the PAS-positive material in the dictyosomes that can already be seen clearly in the confused stage of the primary spermatocytes. There is, however, a subsequent increase in the amount of this material in the spermatids, as Figs. 1–10 show.

As indicated earlier, the increased growth that characterizes the nuclear sap, nucleolus and cytoplasm of cells in the third and fifth lobes of the testis is also shown in the acrosome of those lobes. Indeed these acrosomes attain extraordinary dimensions, a fact which is reflected in the measurements of the PAS-positive material which they contain. As Table 1 shows, the quantity of polysaccharides in the acrosomes of these large spermatids is approximately 40 times as great as that carried in the acrosomes of normal-sized spermatids. The advantages offered by these greater dimensions to cytological and cytochemical analysis are obvious.

An attempt was made to analyze further the chemical nature of the PAS-positive material in the acrosome, the results being presented in Table 2. From the first two rows in this table it is evident that the chemical groups in the acrosomal material which react with the Schiff

Table 1. *Comparison of amounts of polysaccharides (periodic acid Schiff reaction) in the acrosome of large and normal sized early spermatids of Arvelius albopunctatus by microscopic photometric measurements.*

Lobe of Testis	Number of Cells Measured	Acrosome		
		Mean Diameter in Microns	Mean Extinction	Polysaccharides Mean Amount in Arbitrary PAS Units per Acrosome
Third (large sized cells) . .	10	4·75	1·0 ± 0·02	5·76 ± 0·16
Sixth (normal sized cells). .	10	0·76	1·0 ± 0·03	0·14 ± 0·04

Table 2. *Effects of various reagents and enzymes on the polysaccharides (as demonstrated by the periodic acid Schiff reaction) of the dictysomal and acrosomal material of the germ cells of the testis of Arvelius albopunctatus.*

Reagent	Concentration	Time of Exposure	Temperature	PAS Reaction
Acetic anhydride + Pyridine	13 cc. 20 cc.	45 min.	Room	Negative
Acetic anhydride + Pyridine followed by KOH	13 cc. 20 cc. 0·1 N	45 min.	Room	Positive
Methanol Chloroform	1:1	24 hrs.	60° C.	Positive
Saliva	conc.	30 min.	Room	Positive
Amylase (Fisher Scientific) „Amylopsin"	1%	60 min.	37° C.	Positive
Diastase (Merck USP IX)	1%	60 min.	37° C.	Positive
Schering Hyaluronidase bull testis A	4 T.R.U. per 1 cc.	24 hrs.	37° C.	Positive
Schering Hyaluronidase bull testis B	3·3 T.R.U. per 1 cc.	24 hrs.	37° C	Positive
Wyeth Hyaluronidase bull testis	140 T.R.U. per 1 cc. 70 T.R.U. per 1 cc.	24 hrs. 24 hrs.	37° C. 37° C.	Positive Positive
Clostridium welchii Hyaluronidase	—	24 hrs.	37° C.	Positive
Pepsin	0·1 mg. per 1 cc. 0·01 NHCl 5 mg. per 1 cc. 0·01 NHCl	5 min. 30 min.—1 hr.	37° C. 37° C.	Positive Positive

reagent, after periodic acid oxidation, are aldehydes derived from a 1,2 glycol grouping of carbohydrates. This conclusion is substantiated by the reversible acetylation technique in tissue sections, as described by McMANUS and CASON ('50). The acetylation of the 1,2 glycols by acetic anhydride prevents the formation of aldehydes after periodic acid oxidation and thus gives a negative PAS reaction, as seen in the first row of Table 2. The removal of the acetyl groups from the acetylated 1,2 glycols by 0·1 N KOH restores the 1,2 glycol grouping and thus

allows, after periodic acid oxidation, the formation of aldehydes, which color with the Schiff reagent, as seen in row 2 of Table 2. That the positive PAS reaction is actually due to 1,2 glycols of carbohydrates and not to similar groups of glycolipids is shown by the experiments recorded in the third row of Table 2; here the method devised by GERSH ('49) showed that an extraction of glycolipids with hot methanol chloroform resulted in no effect on the positive PAS reaction. Furthermore, it is obvious from Table 2 that the polysaccharide content of the dictyosomal and acrosomal material is not due to the presence of starch or glycogen, because pretreatment of the cells with amylase, diastase or saliva did not change the positive PAS reaction. Control slides containing glycogen in liver cells, fixed and treated in the same manner as the testis slides of *Arvelius*, showed a negative PAS reaction of the glycogen granules after diastase and saliva treatment.

The possibility that the polysaccharide involved is hyaluronic acid was tested by treatment with hyaluronidase. But different types of hyaluronidase, even in concentrations as high as 140 turbidity units (T.R.U. per 1 cc.) which readily digested the hyaluronic acid of umbilical cord, did not affect the PAS reaction of the acrosomal carbohydrates. These results more or less exclude the presence of hyaluronic acid in the acrosome and dictyosomal material of the germ cells of the *Arvelius* testis, although the possibility must be admitted that due to species specificity, the bull testis and bacterial hyaluronidases, which were used in the experiment, do not act on insect hyaluronic acid, or that alternatively the substrate after fixation with Carnoy may be present in a form in which the enzyme is unable to attack it.

Since GERSH and CATCHPOLE ('50) reported the disappearance of the positive PAS reaction in the Golgi material of duodenal cells of the guinea pig after treatment with pepsin we employed this method also on the *Arvelius* material. It was found that a marked decrease of PAS-positive material occurred in the ground substance of the connective tissue lining the lobes of the testis treated with a solution of 5 mg. pepsin per 1 cc: 0·01 N HCl for 30 minutes at 37° C. But in marked contrast to this finding, the PAS-positive reaction of the acrosome showed no detectable change after treatment with GERSH's solution of 0·1 mg. of pepsin per 1 cc. 0·01 N HCl, nor with a concentration of pepsin 50 times as great, applied for 30 minutes (last two rows of Table 2).

In order to determine whether the acrosomal material after Carnoy fixation contains proteins (perhaps in the form of carbohydrate proteins), in addition to polysaccharides, the Millon test and Fast green staining were applied to sectioned material. The results are listed in Table 3.

It is evident from these data that the acrosome unquestionably contains proteins and that the acrosome of the large spermatids has

Table 3. *Comparison of the amounts of proteins (Millon, Fast green) in the acrosome of large and normal sized early spermatids of Arvelius albopunctatus, by microscopic photometric measurements.*

Lobe of Testis	Number of Cells Measured	Acrosome				Ratio between Fast green and Millon
		Mean Diameter in Microns	Stain	Mean Extinctions	Proteins Mean Amounts in Arbitrary Units $r^3 \times E$	
Fifth (large sized) cells	10	4·8	Fast green	·950 ± ·066	5·4 ± 0·4	12:1
Fifth (large sized) cells	10	4·8	Millon	·080 ± ·004	0·46 ± 0·02	
Sixth (normal sized)	10	0·7	Fast green	·920 ± 064	0·11 ± 0·0007	

approximately 40 times as much protein material staining with Fast green as the acrosome of normal-sized spermatids. The latter fact indicates that the increase in proteins corresponds rather closely to the increase of polysaccharides in these huge acrosomes. However it should be noted that if the protein is computed on the basis of extinctions after the Millon reaction, instead of after Fast green staining, a considerably smaller amount is found. Since Fast green is a measure of the basic diamino acids present (arginine, histidine, and lysine) while the Millon reaction involves the tyrosine content, the indications are that we are dealing chiefly with proteins that are very basic.

The ratio between the amounts of protein in the acrosome revealed after Fast green staining and after the Millon reaction is especially striking when compared with the corresponding ratio obtained in the Nebenkern on the same slides (Table 4).

In view of the fact that a close relationship between Golgi material and mitochondria has been postulated by a great many workers (HIRSCH, '39), the mitochondrial Nebenkern was subjected to tests similar to those used for the acrosome. The high extinctions after both Fast green and the Millon test show that the concentration of proteins in the Nebenkern is high (Table 4). But it is also clear from this table that the Fast green: Millon ratio of the Nebenkern (2:1) differs markedly from the corresponding ratio in the acrosome (12:1), thus indicating that the acrosomal protein is more basic in character than the Nebenkern protein.

From the weak Millon and strong Fast green reactions of the acrosome it might be supposed that the Fast green stain of the acrosome is actually due to amino groups of the polysaccharides and not due to amino groups of the proteins. But the disappearance of the PAS reaction after acetylation in the acrosome speaks against the presence of amino groups of the polysaccharides and supports the view that the

Table 4. *Comparison of the amounts of proteins (Millon, Fast green) in the Nebenkern of large and normal Sized spermatids of Arvelius albopunctatus by microscopic photometric measurements.*

Lobe of Testis	Number Measured	Nebenkern				Ratio between Fast green and Millon
		Mean Diameter in Microns	Stain	Mean Extinction	Proteins Mean Amount in Arbitrary Units $r^2 \times E$	
Fifth (large sized) cells	10	10·3	Fast green	·740 ± ·040	19·6 ± 1·20	1·9:1
Fifth (large sized) cells	10	10·3	Millon	·400 ± ·020	10·6 ± 0·53	
Sixth (normal sized) cells . .	10	6·8	Fast green	·720 ± ·036	8·3 ± 0·41	2:1
Sixth (normal sized) cells . .	10	6·8	Millon	·390 ± ·016	4·5 ± 0·18	

Fast green stain in the acrosome is due to the presence of diamino groups of proteins.

In further elaboration of the differences between the Nebenkern and acrosome, the effect of pepsin is to be noted. The proteins of the Nebenkern are readily digested, a fact which stands in decided contrast to the resistance of the acrosomal proteins to such treatment. Finally, the Nebenkern proved to give an entirely negative PAS reaction which showed that, unlike the acrosome, it carries no appreciable amount of polysaccharide.

The chemical evidence would therefore seem to argue against any close relationship between Golgi material and mitochondria, at least as they are represented in the acrosome and the Nebenkern.

V. Discussion.

A. Cytochemistry.

The results of the cytochemical analyses of the acrosome and the Nebenkern may be summarized as follows:

a) The dictyosomal material and the acrosome contain polysaccharides with a 1,2 glycol grouping. There is about 40 times more of these polysaccharides in the acrosome of the large-sized cells than in the normal sized ones.

b) Besides the polysaccharides there is a low but significant protein component in the acrosome which is proportionately larger in amount in the large-sized acrosome as compared with the normal-sized one.

c) In contrast to the acrosome, the Nebenkern of the spermatids does not contain polysaccharides with 1,2 glycol grouping, as is indicated by the negative PAS reaction. On the other hand, our tests indicate considerable amounts of proteins. The quantity of these is proportional to the size of the Nebenkern, so that in the large-sized cells the total quantity of proteins stands in a direct relation to the increased dimensions of the Nebenkern.

d) The protein of the Nebenkern is easily digested by pepsin, in contrast to the protein of the acrosome, and furthermore shows a difference in the Fast green–Millon ratio from that of the acrosome.

The first question which arises is as to the possible chemical nature of the substances responsible for the positive PAS reaction in the dictyosomal and acrosomal material. That the material present in the acrosome is a polysaccharide with a 1,2 glycol grouping seems well established in view of the fact that it was possible to block the 1,2 glycol groups by acetic anhydride—thus giving a negative PAS reaction in spite of periodic acid oxidation—and to unblock the 1,2 glycol groups by the use of KOH (Table 2, rows 1–3). Since glycogen is one of the substances present in tissues and since it also is a polysaccharide with 1,2 glycol groups, its exclusion as an acrosomal constituent responsible for the positive PAS reaction is significant in our considerations. Similarly, the presence of hyaluronic acid (another polysaccharide with 1,2 glycol grouping) in the acrosome can be considered as more than unlikely in view of our enzyme experiments based on the use of hyaluronidase (Table 2, rows 7–10). As already pointed out, it is conceivable that after Carnoy fixation the linkage of the carbohydrate to the protein component of hyaluronic acid in the acrosome may be so firm that the enzyme cannot attack the substrate. However, the fact that the hyaluronic acid of the umbilical cord is readily digested by hyaluronidase after Carnoy fixation argues strongly against this conception.

In an attempt to characterize the substance in the acrosome which yields polysaccharides with 1,2 glycol groups, the simultaneous presence of low amounts of proteins (see Table 3) in the acrosome seems of importance. To our knowledge, the present study demonstrates for the first time a protein component of the acrosome by means of a direct cytochemical protein reaction in situ. GERSH, who, in 1949, reported on a protein component of the Golgi apparatus in the mucosa of the duodenum of rabbit and guinea pig, used in his studies the PAS reaction in combination with proteolytic enzymes. Since he found that the PAS reaction of the Golgi apparatus was abolished after pepsin and trypsin he postulated the presence of a protein (glycoprotein) on the basis of these indirect methods. In this connection the different behavior toward pepsin on the part of the acrosomal polysaccharides as compared with the polysaccharides in the Golgi material merits consideration. In contrast to GERSH, who reported the disappearance of the PAS-positive reaction of the Golgi material after pepsin treatment, we observed no appreciable decrease of the PAS reaction in the acrosome, even after the use of pepsin in a dosage 50 times as strong as that used by him as well as after a longer time of digestion. Since GERSH used the freeze-drying technique for fixation, while in the present study Carnoy was employed as a fixative, the unchanged PAS reaction in the acrosome

after pepsin was first thought to be due to the alteration of the protein by Carnoy, perhaps in such a way that pepsin cannot act on it. But, another explanation seems warranted if we consider that in the same slides in which the PAS-positive reaction was unaltered by pepsin, the PAS-positive reaction of the ground substance of connective tissue lining the testicular lobes disappeared or was markedly decreased. It is possible that the ground substance and the Golgi material in somatic tissues have a protein component which is much more easily digestible by pepsin than the protein component in the acrosome. The loss of the positive PAS reaction in these first structures may then be due to the secondary depolymerizing action and loss of the polysaccharides from the cellular material after the union of the carbohydrate-proteins is split by pepsin.

That the acrosomal protein seems very refractory to pepsin digestion in contrast to other tissue proteins is also evident if the Millon and Fast green reactions are compared in pepsin treated and control slides. The fact that after pepsin treatment the Fast green stains the acrosome just as intensely as in untreated preparations stands in marked contrast to the behavior of the cytoplasm, nucleolus, nucleus, and Nebenkern. The staining, due to Fast green or the Millon reaction, is markedly decreased or completely negated in all these structures after digestion with pepsin. Indeed this differential susceptibility can be utilized in a cytological demonstration of the dictyosomes. After Fast green, in untreated pepsin buffer controls, the dictyosomes are indistinguishable from the uniformly green, cytoplasmic background, whereas after pepsin treatment they retain the Fast green stain and stand out sharply against the cytoplasm which becomes clear and colorless. It is of interest to note that besides the acrosomal proteins, the proteins of the chromosomes and, to a lesser degree, those of the nucleolus, also show resistance to pepsin digestion, which may indicate proteins of a similar composition.

On the basis of the cytochemical findings it seems probable that the material of the acrosome contains a polysaccharide-protein complex which is very similar or even identical with mucopolysaccharides. According to Stacey ('46) mucopolysaccharides though predominantly carbohydrate in nature, have a low but significant protein content. This is a characterization which is also applicable to the acrosome material. On the other hand, the presence in the acrosome of mucoproteins can be excluded. Mucoproteins also represent polysaccharide-protein complexes, but these are predominantly protein in constitution and have such a low carbohydrate content that they would give only a very weak PAS reaction, if any at all.

B. Function of the Acrosome.

The enzymatic nature of at least some of the acrosomal constituents is suggested by the work of several different investigators. Thus, by

means of the phase contrast microscope, AUSTIN ('50) was able to follow the behavior of sperm during the process of fertilization in the rat egg. It was possible to observe the change of the nuclear portion of the sperm head into the male pronucleus, and later its conjugation with the female pronucleus. The acrosome, however, does not participate in these alterations and remains unchanged in the interior of the egg for a considerable length of time. Its role is therefore, quite possibly, that of a catalyst and its substance is not dissipated in the fertilization process.

An acrosomal enzyme is suggested by WISLOCKI ('49) in his work on various tissues of the deer. He reports that the thin cap of the sperm contains alkaline phosphatase.

LEBLOND ('50) suggests that the polysaccharides in the rat acrosome serve primarily, because of their sticky character, to attach the sperms to the Sertoli syncytium. Prior to their transfer to the vasa efferentia however, the sperms are freed again and this LEBLOND attributes to some enzymatic action.

In his work on the sperms of the Japanese beetle, *Pompilia japonica*, ANDERSON ('50) returns to the older suggestions of GROBBEN ('99) and MONTGOMERY ('11) that the cyst-cells in the insect testis function in the nutrition of the sperms. ANDERSON supports a further suggestion of MONTGOMERY that the acrosome acts as an intermediary in the transfer of nutrient substances from the cyst-cells to the sperms. He finds that the acrosome at the top of the sperm becomes imbedded in the cyst-cell, and that it shows alkaline phosphatase activity. This he interprets as indicating the transfer of nutrition materials from the cyst-cell to the sperm, and if he is right we have here a process in which the acrosomal substance thus acts as an enzyme. However, the transfer of nutritive materials is perhaps of limited importance since, in most cases, the sperms have attained full growth prior to attachment, even though elongation may continue. Therefore, the suggestion of STERN and HADORN ('38) that such attachment may be necessary to induce mobility in the ripe sperms should also be considered.

In this connection, the possible role of the acrosome in the process of fertilization merits more extensive discussion. Earlier workers, among them BOWEN, suggested that it might function in the penetration and activation of the egg, and he pointed out the close analogy between the formation of the acrosome and that of a secretory granule. Extending this analogy he suggested that the Golgi material in the acrosome may be a center for the formation of enzymes which may play a part in the activation of the egg. The possible existence of enzymes in the sperm and their importance for the process of fertilization had already been stressed by LILLIE and LOEB.

Recent work has shown that male germ cells of all species examined contain an enzyme hyaluronidase which dissolves the cementing ma-

terial surrounding the female germ cells and thus makes fertilization possible (FEKETE and DURAN-REYNALS, '43; MCCLEAN and ROWLANDS, '42; ROWLANDS, '44). Exceptions are the male germ cells of reptiles and birds in which hyaluronidase has not been found and where, accordingly, the ova not surrounded by follicle cell cumuli (JOËL, '42; KURZROCK, LEONARD and CONRAD, '46).

The presence and possible role of the enzyme hyalurinodase in the acrosome and its elaboration by the dictyosomal material of the spermatocytes therefore demands consideration. The studies of RIISFELT ('49) demonstrating that during rat spermatogenesis the hyalurinodase is first found in the spermocytes may be a corollary to our findings of the appearance of the dictyosomal material in the primary spermatocytes and suggests possible relationship between dictyosomal material and hyaluronidase. Whether the 1,2 glycol grouping of the polysaccharides in the dictyosomal material and the acrosome is indicative of the presence of hyaluronidase itself must await further chemical characterization of the enzyme. Studies in our laboratory, in which bull testis hyaluronidase was tested in vitro for 1,2 glycol groups, gave a positive PAS reaction. Moreover, a series of preparations of this enzyme, containing respectively 220, 550, 900 and 1400 T.R. units per mg showed a corresponding increase in the intensity of the PAS reaction. Since, according to HOTCHKISS, the amount of dye fixed is dependent upon the actual weight of glycol structure present, it seems that the more purified the enzyme preparation (for instance 1400 T.R.U. per mg as compared with 200 T.R.U. per mg), the more 1,2 glycol groups can be demonstrated by means of the PAS reaction. Whether the 1,2 glycol groups are actually a part of the chemical constitution of the enzyme hyaluronidase, or whether they happen to be an "impurity" which became more concentrated during the purification process of the enzyme, must await the testing of enzyme preparations with higher T.R.U. per mg, which are not yet available.

The possibility that the enzyme hyaluronidase contains 1,2 glycol linkage and would thus give a positive PAS reaction if present in tissues, led us to investigate the sperm of a snake in which as previously mentioned, no hyaluronidase has been found, and to compare it with the bull sperm, which serves as a good source for the extraction of hyaluronidase. While both sperms show a distinct acrosome, the sperm of the snake, *Natrix sipedon confluens*, showed at most only a very slight amount of PAS-positive material at the extreme tip of the acrosome (Fig. 18), in contrast to the bull sperm where the whole acrosome, which consists of a thin hull covering two thirds of the sperm head, was stained by the PAS reaction (Fig. 17). That the PAS-positive reaction in the acrosome of the bull sperm is not due to starch, glycogen or hyaluronic acid was shown by pretreating sections with amylase,

diastase and bull testis hyaluronidase without any effect on the intensity of the PAS reaction. In brief, the snake sperm which, in chemical determinations, has failed to show hyaluronidase, is furnished with only a minimal amount of PAS-positive material while the sperm of the bull, from which a copious supply of hyaluronidase can be derived, carries a considerable quantity of the substance that stains in the PAS reaction.

But while this correlation, as well as some of our other findings, may be significant in establishing an association of the enzyme hyaluronidase with the *Arvelius* acrosome, the nature of this association is not clear. No definite conclusions can be drawn as to the active groups of the enzyme, and the findings concerning the 1,2 glycol groups in the present stage in no way imply that these are the prosthetic groups of the enzyme. But whether or not we deal here with the enzyme itself, the presence of mucopolysaccharides in the acrosome may be suggestive for further elucidations of the problems of fertilization and sterility.

C. Sterility.

Occasional irregularities which influence the effectiveness or viability of some of the germ cells are encountered in the testis of all species. These are of an accidental nature (as for instance mishaps to the mitotic apparatus) and are negligible in causing sterility of an individual. A more general detrimental effect may be produced by physiological changes in an individual, and then of course all the cells in a gonad may become nonfunctional.

But in all such cases, only the individual is affected. Departures from the normal spermatogenesis which occur regularly in a certain stock, must have a genetic basis. But the recognition of that fact offers scant help in elucidating the more immediate physiological causes of such deviation. A somewhat closer approach to an analysis seems to be offered by certain pentatomid species like *Arvelius*. They are especially interesting in that they offer cases in which these deviations differ widely in their manifestations, even though everything points to the fact that the underlying physiological factors differ only slightly from the normal ones.

For one thing, these conditions are encountered in all males of certain species. They are moreover restricted to definite sections or lobes of every testis and are correlated with departures from the normal spermatogenesis which are always the same in a given species. Such species may be put into two groups:

In one group all the species are characterized by deviations in the maturation process which occur in all the cells of a single lobe of the testis. The cytological deviations are so bizarre that the lobe concerned has been called the "harlequin lobe." In effect they comprise a failure on the part of the chromosomes to form bivalents, and special mitotic developments which result in an unequal distribution of the chromo-

somes (SCHRADER, '46a, '46b). Sperms seem to be formed from most of such cells nevertheless, but it is almost certain that few of them are functional in normal fertilization. This is adduced from the fact that specimen with abnormal chromosome numbers have not yet been encountered in the field, though 12 species are now known to have harlequin lobes and some 50 specimen have been examined. More extensive collecting would be necessary to determine whether the incidence of individuals with abnormal chromosome numbers is higher for these species than for species without harlequin lobes. But in effect, the harlequin lobe seems to constitute a sterile section of the testis.

Another and much larger group is represented by *Arvelius*. Here the spermatocytes of some lobes show a different amount of growth than the spermatocytes of adjoining lobes. But as already stated, these differences in synthetic activity do not affect the chromosomes which are identical in size in the different lobes and undergo a normal meiosis (SCHRADER and LEUCHTENBERGER, '50). Since the chromosomes are not affected, it is possible that functional sperms are formed unless the deviations in the size of other cell elements are extreme, as they are in the third and fifth lobes.

However, it is pertinent to point out that though it was shown that protein and DNA synthesis may proceed independently of each other, it developed on the other hand that the synthesis of RNA and proteins are closely linked. This explains the proportionate increase of cytoplasm and nucleoplasm while the chromosomal material remains constant.

The present study demonstrates that the synthesis of acrosomal material may in turn exhibit a certain degree of independence of the synthesis of all the other cell structures already named. The physiological changes in the third and fifth lobes which bring about a great increase of nucleoplasm, cytoplasm and nucleolus, induce an even more marked growth of acrosomal material. The result is an enormous acrosome which quite possibly affects the efficiency of the sperm in fertilization. This disproportionate increase implies that acrosomal synthesis is affected more easily by alterations in the physiological basis than the synthetic processes of the other cellular elements. It is conceivable that slightly different changes in the testicular conditions may bring about an insufficiency instead of a superabundance of acrosomal material and this may indeed be even more detrimental to the role of the sperm in fertilization. In short, there is here the suggestion that very slight alterations in the physiological environment may affect the formation of the acrosome and hence bring about some degree of sterility. Studies, now in progress, concerned with the problem of sterility in the human male seem to indicate that there is an optimum amount of acrosomal material which is necessary for the process of fertilization.

In both types of abnormality, chromosomal as well as acrosomal, the affected lobes of the testis lie in the midst of lobes which produce

perfectly normal sperms. The observable, structural conditions seem to be identical for all the lobes of a testis and yet it is obvious that local differences that alter some of the physiological processes in these lobes, must be present. But the localized sterility, which probably results in both groups, does not greatly influence the life of the affected males. They are just as vigorous and active as are the males of other species and the very fact that such species survive indicates that adaptation to these peculiar reproductive abnormalities has been attained.

It is not likely that such deviations from the normal conditions are confined to the pentatomids. These cases merit special attention because they show that widely different types of sterility may result from slight alterations in the structure and physiology of the testis. Chromosomal irregularities thus induced, obviously must affect the role played by the fertilizing sperm. But the case of *Arvelius* shows that non-chromosomal effects may be just as important, even though confined to the process of fertilization itself. If, as we suggest, the acrosome carries an enzyme that is necessary for the initiation of development, slight changes in the formation of acrosomal material may greatly influence the effectiveness of the sperm. Since the production of this material appears to be more readily altered (at least quantitatively) than is the synthesis of proteins and DNA, it is quite possible that here is represented a very common cause of sterility.

However, the question arises of why such constantly occurring departures from more normal sperm conditions should persist in so many species and why they should not have been eliminated in the course of evolution. The implication that these excessive quantities of non-chromosomal constituents of the cell may confer certain advantages can not be lightly discarded, especially where the acrosome is concerned.

As has already been mentioned, the mucopolysaccharides, which are present in the acrosome, are substances of a "sticky" nature and may function in the attachment of the sperm to the egg. But the role of the individual acrosome may further be important in gaining access of the sperm to the interior of the egg and it is in this connection that acrosomal enzymes may well play a role. It is also not unlikely that the sperm entry is facilitated by the additional enzymes that might be contributed by supernumerary sperms. This may indeed explain the prevalence of polyspermy in many groups of animals (in insects it appears to be almost universal) and the giant acrosomes of such pentatomids as *Arvelius* would represent especially copious sources of enzymes. In short, natural selection may actually favor such special developments as are encountered in the third and fifth testicular lobes of *Arvelius*.

Such a consideration may throw light on the observation that a sparsity of mobile sperms in the human seminal fluid is correlated with male sterility. Since only a single sperm is involved in the final

union of pronuclei, which constitutes the successful culmination of the fertilization process, and since the seminal fluid even of sterile males frequently shows many more motile sperms than there are mature eggs in the female, it may well be asked why a still greater abundance of sperms is necessary for normal fertility. A possible answer is implied in the preceding paragraph—that the additional quantity of enzymes furnished by supernumerary sperms promotes sperm entry. Possibly such advantageous effects become noticeable only when the total of such enzymes exceeds a certain minimal quantity.

Summary.

1. The study of certain structures, like the acrosome and Nebenkern, in the developing sperms of *Arvelius* is facilitated by the fact that they are present in greatly enlarged dimensions in certain testicular lobes while retaining normal size in adjoining lobes.

2. The dictyosomes or Golgi bodies in the spermatocytes are stained red by the PAS treatment. They are assembled to form the similarly stained acroblast in the spermatid, and the acrosome that is finally evolved is also PAS-positive in its color reaction. All the evidence indicates that in the process of acrosome formation there is a direct utilization of dictyosomal material which is already present two cell generations earlier.

3. The substance responsible for the red staining of the dictyosomes and acrosome is a polysaccharide with a 1,2 glycol grouping. There is also direct evidence for the presence of small quantities of proteins which probably form a complex with the polysaccharides. This complex is similar or perhaps identical with a mucopolysaccharide.

4. The Nebenkern, which is a mitochondrial derivative, does not contain polysaccharides with a 1,2 glycol grouping. On the other hand, it contains considerable amounts of proteins which, in contrast to those of the acrosome, are easily digested by pepsin. The evidence does not support the suggestion, frequently made, that the acrosome and Nebenkern are closely interrelated.

5. The enzymatic nature of the acrosome and its possible role in fertilization and sterility are discussed.

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DAS VORKOMMEN VON KOHLENHYDRATEN IM RUHEKERN UND WÄHREND DER MITOSE.

Von

HANS STICH*.

Mit 7 Textabbildungen.

(Eingegangen am 15. Mai 1951.)

Bis vor kurzem war über das Vorkommen von Kohlenhydratverbindungen im Kern nur die Anwesenheit von Ribose und Ribodesose in den Nukleinsäuren bekannt, wobei nur die Ribodesose cytochemisch durch die Feulgenreaktion nachgewiesen werden konnte. Mit der bereits seit längerem verwendeten Zuckerreaktion nach BAUER (1933a) ließen sich keine anderen Zucker — sei es als Polysaccharide oder als Glykoproteide — auffinden. Das wurde erst möglich mit dem von HOTCHKISS (1948) angegebenen Nachweis für Kohlenhydrate, der darauf beruht, daß durch Behandlung mit Überjodsäure in den Kohlenhydraten zwei benachbarte CHOH-Gruppen unter Bruch ihrer Bindung zu Aldehydgruppen CHO oxydiert werden und sich die letzteren durch fuchsinschweflige Säure färberisch nachweisen lassen. Diese Reaktion, deren chemische Natur vollkommen bekannt ist, ist wesentlich empfindlicher und in der Lokalisation präziser als die Bauersche Methode, bei der auch die Art der Oxydation durch Chromsäure nicht bekannt und vermutlich nicht spezifisch für bestimmte Bindungen ist.

Zur Untersuchung des Zellkerns ist die Hotchkiss-Methode zuerst von MONNÉ und SLAUTTERBACK (1950) benutzt worden, die in frühen Entwicklungsstadien des Seeigels *Paracentrotus lividus* eine positive Reaktion an Interphasekernen, Spindeln und Centrosomen erhielten. Um einen vorläufigen Überblick über das Vorkommen von Hotchkiss-positiven Kohlenhydraten zu erhalten, erschien es wichtig, Kerne zu untersuchen, bei denen ein unterschiedlicher Stoffwechsel erwartet werden kann, nämlich einerseits aus Zellen, die eine intensive Eiweißsynthese aufweisen, und andererseits Kerne aus Teilungsgeweben und von sich teilenden Zellen. Stoffliche Verschiedenheiten konnten hier mit einiger Berechtigung erwartet werden, da sich diese beiden Kerntypen in morphologischen (Kernsaftreichtum, Ausbildung von Nukleolen und Nukleoleneinschlüssen, Kernmembranfaltung) und physiologischen

* Mit Unterstützung durch die Notgemeinschaft der Deutschen Wissenschaft.

Merkmalen (Empfindlichkeit gegen kurzwellige Strahlungen und Mitosegifte), sowie in ihrem Stoffbestand (z. B. Eiweiß-, Nucleinsäure- und Phosphatasegehalt) unterscheiden.

Material und Methoden.

Als Zellen mit reichlicher Eiweißproduktion wurden gewählt: Ganglienzellen, für die HYDÉN (1943) den starken Eiweißumsatz analysiert hat, Leberzellen, deren Eiweißstoffwechsel von STOWELL (1947) untersucht worden ist, wachsende Oocyten, die CASPERSSON (1940) herangezogen hatte, und wachsende *Acetabularia*-Zellen (STICH 1951). Von sich teilenden Zellen wurden Oogonien, Oocyten und Furchungszellen untersucht.

Als Objekte wurden verwendet: *Acetabularia mediterranea*, *Ascaris megalocephala* (Eireifung und Furchung), *Cyclops strenuus* und *Diaptomus castor* (Oogonien, wachsende Oocyten, Eireifung und Furchung), *Tipula oleracea* (wachsende Oocyten), weiße Mäuse (Ganglien, Leber und Niere) und Haustauben (Ganglien).

Die Kohlenhydrate wurden nach der Vorschrift von HOTCHKISS dargestellt[1]. Eine weitere Prüfung zur näheren Kennzeichnung der chemischen Natur der reagierenden Substanzen, wie sie LEUCHTENBERGER und SCHRADER (1950) durch Verwendung verschiedener Reagenzien und Enzyme vorgenommen haben, war mir bisher nicht möglich.

Zur Fixierung, die stets bei Zimmertemperatur (18—22° C) erfolgte, wurden verwendet: Bouin-Allen (40—60 min), Carnoy (10—20 min), Petrunkewitsch (40 bis 60 min), Zenker (40—60 min) und 75—85% Äthylalkohol (2—3 Std). Die Intensität der Farbreaktion variierte etwas nach Verwendung der verschiedenen Fixierungsmittel. Da aber keine exakten Messungen des Reaktionsausfalles möglich waren, konnte diese Erscheinung in Kauf genommen werden.

Nach dem Vorgang von MONNÉ und SLAUTTERBACK wurde dort, wo infolge starker Reaktion von Dotter und anderen Plasmaeinschlüssen die Kerne verdeckt wurden, nach der Anweisung von ALBERT und LEBLOND ein Teil der freigelegten Aldehydgruppen vor der Reaktion mit fuchsinschwefliger Säure durch 2-4-Dinitrophenylhydrazin blockiert, wodurch diese Substanzen einen (nicht leicht zu beurteilenden) gelben Farbton annahmen zum Unterschied von den nichtblockierten, die durch die fuchsinschweflige Säure rotgefärbt wurden.

Um in Hotchkiss-Präparaten auch die Chromosomen sichtbar zu machen, wurde mit Methylgrün nachgefärbt, das nach der Methode von KURNICK (1950) von Methylviolett gereinigt und verwendet wurde. Man erhält so eine selektive Färbung der Desoxyribonukleinsäure (POLLISTER und LEUCHTENBERGER 1949) und mithin der Chromosomen, die sich in grünem Farbton von dem Rot der Spindeln und Astrosphären gut abheben.

Vorkommen von Kohlenhydraten in Kernen von Zellen mit starker Eiweißsynthese.

Im Kernsaft und in den Chromosomen von Oocytenkernen (*Cyclops strenuus*, *Diaptomus castor*, *Tipula oleracea*, *Ascaris megalocephala*), von motorischen Ganglienzellen (Maus, Taube) und von Leber- und Nierenzellen erwachsener Mäuse ließen sich keine Kohlenhydrate nach-

[1] Herr Prof. T. CASPERSSON stellte die Überjodsäure (ein Fabrikat der Frederick G. Smith Co., Columbus, Ohio) Herrn Prof. H. BAUER zur Verfügung, von dem ich sie erhielt.

weisen. Nur bei *Acetabularia mediterraneà* zeigte der in Form eines Netzwerkes fixierte Kernsaft eine schwache Reaktion.

Demgegenüber ergab der Nukleolus eine zwar oft nur recht schwache, doch stets deutlich erkennbare Färbung, wie dies auch von MONNÉ und SLAUTTERBACK (1950) für die Nukleolen von Oocytenkernen von *Paracentrotus lividus* angegeben wird. Die Kohlenhydrate ließen sich nur in der aus Eiweißstoffen, Nukleinsäuren und Phosphatasen bestehenden eigentlichen Nukleolarsubstanz nachweisen, nicht jedoch in den tropfigen oder blasigen Nukleoleneinschlüssen (Nukleolen der Oocytenkerne von *Cyclops*, *Diaptomus* und des Kerns von *Acetabularia*). In den Oocytenkernen von *Tipula* ist die ribonukleinsäurehaltige Nukleolarsubstanz diffus im ganzen Kernraum verteilt und kann durch Zentrifugieren (20 min mit etwa 1900 × g) an dem zentrifugalen Pol des Kernes als Kalotte zusammengeballt werden (BAUER 1933 b). Dementsprechend fällt auch die Hotchkiss-Reaktion in normalen Oocytenkernen diffus aus und beschränkt sich in zentrifugierten auf den zentrifugalen Kernpol.

Um zu entscheiden, ob in den Nukleolen wirklich eine cytochemische Reaktion in situ vorliegt, wurden an *Cyclops*-Oocyten einige Versuche unternommen:

1. Wurde die Überjodsäurebehandlung der Schnittpräparate weggelassen, die einzelnen Reaktionsschritte der Hotchkiss-Methode jedoch wie üblich ausgeführt, so blieben wie alle anderen kohlenhydrathaltigen Zellteile auch die Nukleolen vollkommen ungefärbt. Es handelt sich also nicht um Färbung mit zersetzter fuchsinschwefliger Säure.

2. HOTCHKISS fand an reinen Substanzen, daß Ribose eine positive Reaktion ergibt, daß sie jedoch im Nukleinsäuremolekül keine Aldehyde nach der Überjodsäurebehandlung bildet; um diese Erfahrung auch auf Schnittpräparate auszudehnen, wurde einmal vor, das andere Mal nach der Extraktion der Ribonukleinsäure mit selbsthergestellter und mit kristalliner Ribonuklease[1] der Kohlenhydratnachweis geführt. Die Färbung der Nukleolarsubstanz war in beiden Fällen gleich, beruht also nicht auf einer Darstellung des Zuckers in der Ribonukleinsäure.

3. Nach Fixierung mit den oben aufgezählten Fixierungsmitteln, Behandlung mit Überjodsäure und daraufhin Einstellung der Präparate für 24 Std in 2-4-Dinitrophenylhydrazin verloren die Nukleolen die Fähigkeit, mit der fuchsinschwefligen Säure zu reagieren. In fast allen Fällen ließ sich eine Gelbfärbung der Nukleolarsubstanz feststellen. Eine Gelbfärbung tritt nicht ein, wenn die Perjodsäurebehandlung unterlassen wird. Dies weist darauf hin, daß durch die Perjodsäurebehandlung im Nukleolus Aldehydgruppen entstehen, d. h., daß im Nukleolus Kohlenhydrate vorkommen. Dieser Versuch wurde deshalb ausgeführt, um den

[1] Frau Dr. C. LEUCHTENBERGER danke ich herzlich für die freundliche Überlassung von kristalliner Ribonuklease (aus den Armour Laboratories, Chicago).

Einwand auszuschalten, daß die nach der Hotchkiss-Reaktion im Nukleolus auftretende rote Färbung nicht auf der Anwesenheit von Aldehydgruppen in diesem beruht, sondern durch eine Diffusion der Farbstoff-Aldehydverbindung von ihrem Entstehungsorte und nachträgliche Adsorption an der Nukleolarsubstanz verursacht wird.

Diese Tatsachen sprechen wohl dafür, daß in den Nukleolen Kohlenhydrate vorkommen, doch scheint ihre Menge wesentlich geringer zu sein als im Cytoplasma, Dotter oder im Kernsaft sich teilender Kerne.

Vorkommen von Kohlenhydraten in sich teilenden Kernen.

Die Eier und Furchungsstadien von *Cyclops strenuus* waren für den Nachweis der Kohlenhydrate außerordentlich gut geeignet, da die Dotterschollen fast keine Reaktion ergaben und so bereits ohne Behandlung mit 2-4-Dinitrophenylhydrazin die Kernverhältnisse deutlich erkennen ließen.

1. Spindel. Die nach Fixierung und Färbung mit Delafieldschem Hämatoxylin oder Toluidinblau in der 1. und 2. Oocyten-Reifespindel sowie den Furchungsspindeln darstellbaren Spindelfasern geben eine deutliche positive Kohlenhydratreaktion. Die Chromosomen zeigen keine Anfärbung. Abb. 2 und 3 demonstrieren Spindeln der ersten Oocytenreifeteilungen von *Cyclops strenuus*, wie sie auch nach verschiedenen Färbungen (Delafieldschem Hämatoxylin, Feulgenreaktion mit Lichtgrün-Gegenfärbung, Toluidinblau, Gallocyanin oder Methylgrün-Pyrcnin) und auch nach verschiedenen Fixierungsmitteln in fast gleicher Form erscheinen. Die auf den Abbildungen sichtbaren hellen Räume in den Spindeln sind Höfe um die Chromosomen, die in gepaartem Zustand in der Mitte dieser Räume liegen. Die konjugierten Chromosomen — soweit diese an den ausgeführten Färbungen erkannt werden können — liegen nicht dicht aneinander. Zwischen ihnen befindet sich vermutlich eine Substanz, die sich nicht mit basischen Farbstoffen anfärben läßt. Die genaueren morphologischen Verhältnisse dieser Reifespindel, sowie die „Distanzkonjugation" der Chromosomen werden augenblicklich bearbeitet.

2. Centrosomen und Astrosphären. Die Hotchkiss-Reaktion fällt in diesen Zellstrukturen wesentlich intensiver aus als im Cytoplasma (Abb. 6).

3. Interphasenkerne. Im ♂ und ♀ Vorkern lassen sich bereits kurz nach ihrer Entstehung Kohlenhydrate nachweisen. Das kohlenhydrathaltige Material erscheint im Kernsaft in Form von Grana, Stäbchen oder als Netz (Abb. 5 und 6), wobei jedoch ausdrücklich betont sei, daß man diese Struktur kaum als naturgetreu erhalten ansehen darf. Es steht jedoch fest, daß in beiden Vorkernen, sowie in den Kernen aller Furchungsstadien (Abb. 7) das kohlenhydrathaltige Material sich im Kernsaft befindet und nicht im Chromatin.

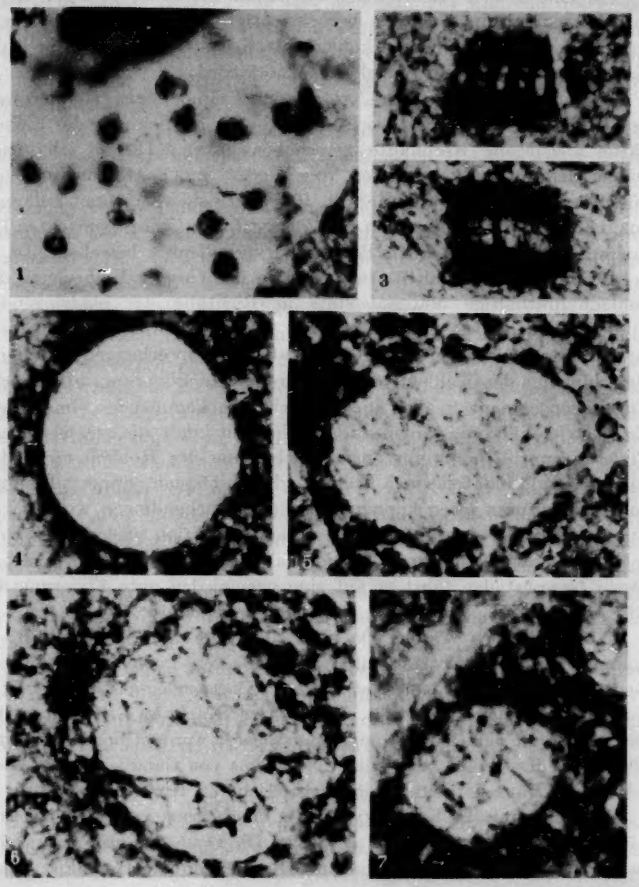

Abb. 1—7. *Cyclops strenuus*. Hotchkiss-Reaktion. Abb. 1—6 1070 ×. Abb. 7 1530 ×. *1* Oogonienkerne. *2, 3* Oocyten. Metaphase I. Die Chromosomen, die keine Reaktion ergeben, liegen in gepaartem Zustand in der Mitte von hellen Räumen der Spindel. Ihre Sichtbarkeit beruht allein auf ihrer starken Lichtbrechung. *4* Kern aus einer erwachsenen Oocyte. Kernraum ohne nachweisbare Kohlenhydrate. *5* Kern aus einer erwachsenen Oocyte kurz vor dem Beginn der 1. Reifeteilung. Im Kernraum ein Netzwerk, das eine sehr deutliche Hotchkiss-Reaktion ergibt. *6* Bereits aneinanderliegende Vorkerne eines ungefurchten Eies. Im Kernraum ein Hotchkiss-positives Netzwerk, links das stark gefärbte eine Centrosom. *7* Kern aus einem 16-Zellenstadium. Im Kernraum ein Hotchkiss-positives Netzwerk.

Für eine richtige Beurteilung der Lokalisation der Kohlenhydrate muß unbedingt die Tatsache berücksichtigt werden, daß eine starke zentripetale Verlagerung des kohlenhydrathaltigen Materials durch die eindringende Fixierungsflüssigkeit bewirkt wird:

In Furchungsstadien von *Cyclops* konnte einige Male gefunden werden, daß die Kohlenhydrate sich innerhalb der Kerne in die der Eioberfläche abgelegene Kernhälfte verschoben haben. Die so erhaltenen Bilder gleichen weitgehend den von MONNÉ und SLAUTTERBACK veröffentlichten (z. B. ihre Abb. 8, 9, 10). Die beiden Autoren schlossen aus ihren an Furchungsstadien von *Paracentrotus lividus* gemachten Befunden, daß in den Kernen Polysaccharidgradienten bestehen. Da in den Kernen von Seeigel-Blastulae und in den *Cyclops*-Embryonen das kohlenhydrathaltige Material stets in Richtung der eindringenden Fixierflüssigkeit lag, scheint mir die gegebene Interpretation nicht zwingend zu sein. Die gefundenen Tatsachen sprechen eher für eine Verschiebung der Kohlenhydrate während der Fixierung, als für das Vorhandensein eines realen, auch im Leben vorkommenden Gradienten. Es besteht allerdings auch die Möglichkeit, daß die verschiedenen Fixierungsmittel nicht nur eine Verschiebung der Kohlenhydrate bewirken, sondern daß sie auch Kohlenhydrate abbauen oder ihre Bindung an Zellstrukturen lösen können. Ähnliche Erscheinungen wurden von LISON (1949) bei Ausführung der Glykogenreaktion nach BAUER vorgefunden. Den ungleichmäßigen Ausfall dieser Reaktion in verschiedenen Gewebeteilen führt er auf eine verschieden gute Fixierung des Glykogens im Gewebezentrum und am Geweberand zurück.

4. Cytoplasma. Werden Oocyten oder reife Eier zentrifugiert, so können Plasma und Dotter auf Grund ihres verschiedenen spezifischen Gewichts voneinander getrennt werden. Dies ermöglicht den exakten Nachweis, daß das Cytoplasma eine positive Hotchkiss-Reaktion ergibt. Dieser Befund darf jedoch nicht generalisiert werden, denn das Cytoplasma der Keimzellen sowie das Cytoplasma von Ganglien- und Leberzellen von Mäusen besitzt keine nachweisbaren Kohlenhydrate.

Das Verhalten der Kohlenhydrate in den Kernen verschiedener Entwicklungsstadien von *Cyclops* wechselt. Die Oogonienkerne geben eine zwar nicht allzu starke, aber doch deutlich erkennbare Hotchkiss-Reaktion (Abb. 1). In den Kernen junger sowie alter Oocyten lassen sich keine Kohlenhydrate nachweisen. Erst in den Kernen ausgewachsener Oocyten, die kurz vor dem Eintritt in die Reifeteilung stehen, beginnt sich im Kernsaft, aber nicht in den Nukleolen oder den Chromosomen, kohlenhydrathaltiges Material anzusammeln. Kurz vor der Reifeteilung ist der Kern gleichmäßig erfüllt von einem kohlenhydrathaltigen Netzwerk (Abb. 5). Während der Ausbildung der Spindel, die hier, wie bekannt, intranucleär entsteht, gehen die Kohlenhydrate in die Spindel-

fasern ein. Diese Erscheinung läßt sich sehr gut während des ersten Teilungsschrittes verfolgen. In den Vorkernen des ungefurchten Eies erscheint das kohlenhydrathaltige Material „netzförmig" angeordnet. In der Prophase erfolgt dann eine Umordnung dieses „Netzwerkes" zu den Spindelfasern.

Auf höheren Furchungsstadien lassen sich 2 Zelltypen scharf voneinander trennen: die somatischen Blastomeren, die sich weiter teilen, und die 2 Urgeschlechtszellen, die während des ganzen weiteren Furchungsverlaufes und der Naupliusdifferenzierung keine Mitosen mehr ausführen. Dieser Unterschied macht sich auch an den Kohlenhydraten bemerkbar. Die somatischen Kerne besitzen ein kohlenhydrathaltiges Netzwerk, während die beiden Kerne der Urgeschlechtszellen keine positive Hotchkiss-Reaktion im Kernsaft ergeben.

Zum Vergleich wurden noch verschieden alte Entwicklungsstadien von *Ascaris megalocephala* herangezogen. Im Gegensatz zu *Cyclops* ergaben verschiedene Plasmaeinschlüsse eine so starke Hotchkiss-Reaktion, daß die Kernverhältnisse nicht beobachtet werden konnten. Aus diesem Grunde mußte die 2-4-Dinitrophenylhydrazin-Behandlung ausgeführt werden. Nach einer Einwirkung von 12—26 Std erschienen einige Plasmaeinschlüsse gelb, andere reagierten noch mit der fuchsinschwefligen Säure und waren deshalb rot, während in den beiden Vorkernen des ungefurchten Eies und in der 1. Furchungsspindel keine sichtbare Farbreaktion eintrat. In solchen Fällen ist es schwierig zu entscheiden, ob die Kerne wirklich keine Kohlenhydrate enthalten oder ob der negative Ausfall nur durch die 2-4-Dinitrophenylhydrazin-Behandlung vorgetäuscht wird.

Die Reifespindeln in den *Ascaris*-Oocyten ergaben stets eine intensive Reaktion. In der Anaphase konnte zwischen den auseinanderweichenden Chromosomen, die selbst keine Reaktion aufweisen, kohlenhydrathaltiges Material nachgewiesen werden.

Diskussion.

Der Kohlenhydratgehalt des Kernsaftes scheint von der Funktion des Kernes abhängig zu sein. Kerne in sich nicht teilenden tierischen Zellen besitzen keine oder nur geringe im Nukleolus konzentrierte Mengen von den mit der Hotchkiss-Reaktion nachweisbaren Kohlenhydraten. In sich teilenden Kernen, soweit sie hier untersucht wurden, hingegen läßt sich ein hoher Kohlenhydratgehalt nachweisen. Dieser befindet sich im Kernsaft und sammelt sich in der Prophase in den Spindelfasern an. Diese Befunde deuten auf eine wesentliche Bedeutung der Kohlenhydrate für den extrachromosomalen Mechanismus der Kernteilung hin. Das Verhalten der Kohlenhydrate in den Kernen von Oogonien, jungen und alten Oocyten demonstriert, wie in *einem* Kern,

je nach der Funktion, die er augenblicklich ausübt, der Polysaccharidgehalt wechseln kann. Ebenso erkennt man, daß in der Blastula so *eng nebeneinander*liegende Kerne wie die von somatischen Blastomeren und von Urgeschlechtszellen — Kerne verschiedener Teilungsbereitschaft — sich in ihrem Kohlenhydratgehalt unterscheiden können.

In diesem Zusammenhang sind die Befunde von BULLOUGH (1949a, b, 1950) und von BULLOUGH und EISA (1950) von besonderem Interesse. Die Autoren fanden, daß in der Haut von Mäusen die Mitosen von der vorhandenen Zuckermenge abhängig sind und daß bei einer experimentellen Steigerung oder Senkung der Kohlenhydrate der Haut auch die Mitosen zu- bzw. abnahmen. Ferner ist es ihnen gelungen, die Mitosephase zu erfassen, die den Zucker benötigt: "Apparently it is only the transition from interphase to the prophase that is sensitive to the presence or absence of glucose or glycogen, and once a division is under way it becomes entirely insensitive" (BULLOUGH and EISA 1950, p. 262). Dieser Befund deckt sich gut mit der obigen Feststellung, daß vor Beginn der Prophase im Kern Kohlenhydrate angesammelt werden. Es bleibt hierbei allerdings noch offen, ob diese im Kern synthetisiert werden oder ob sie aus dem Plasma in den Kern diffundieren.

Mit den oben beschriebenen Befunden sowie mit denen von BULLOUGH stehen die von HUGHES (1950) durchgeführten Versuche mit Mitosegiften in gutem Einklang. Er fand, daß Natriumfluorid und Jodacetat, Stoffe, die Phosphorylisierungsprozesse hemmen, den Eintritt des Kernes in die Prophase verhindern. Eine weitere Schlußfolgerung aus diesen Ergebnissen wäre zur Zeit noch verfrüht, da weder die Zuckerart, die mit der Hotchkiss-Reaktion erfaßt wurde, noch ihre Bedeutung definiert werden können. Es bestehen die beiden Möglichkeiten, daß der Zucker entweder als Energiedonator wirkt oder daß er, vielleicht als Glycoproteid, bei der Bildung von Zellstrukturen eine Funktion ausübt.

Das frühzeitige Auftreten von kohlenhydrathaltigem Material in den Kernen der reifen Oocyten von *Cyclops* läßt erkennen, daß bereits lange vor dem Beginn der Prophase, die man an Auflösung des Nukleolus, Kontraktion der Chromosomen und Spindelbildung erkennen kann, Stoffumsetzungen im Interphasekern ablaufen, die auf die Teilung hingerichtet sind. Auch das Vorhandensein von Kohlenhydraten in den jungen, noch nicht vergrößerten Vorkernen des *Cyclops*-Eies sowie in den Interphasekernen aller Furchungsstadien und im Gegensatz hierzu das Fehlen in den Kernen der Urgeschlechtszellen demonstriert, daß bereits in der Interphase ein Unterschied besteht zwischen Kernen, die sich teilen werden und Kernen, die sich nicht mehr teilen werden. Beide soeben erwähnten Kerntypen unterscheiden sich nicht nur in dem Kohlenhydratgehalt, sondern auch, wie neuere Untersuchungen von

Pasteels und Lison (1950) erkennen lassen, in ihrem Desoxyribonukleinsäurebetrag und in der Größe der P^{32}-Erneuerung der Kernnukleinsäuren (A. Goward und S. Pelc, 1951).

Die Spindeln der Reifeteilungen des *Cyclops*-Eies sowie der niederen Furchungsstadien entstehen, wie bereits oben erwähnt, intranukleär. Die Substanzen, die für die Spindelbildung verwendet werden, müssen infolgedessen spätestens während der Prophase sich im Kernsaft ansammeln und können dort cytochemisch nachgewiesen werden. Auf letzterem Wege gelang auch der Nachweis, daß die Ribonukleinsäure, die in den Spindeln aller bisher daraufhin untersuchten Objekte (*Cyclops*, *Diaptomus* und *Tipula*) vorhanden erscheint, erst in der frühen Prophase sich im Kernsaft ansammelt (Stich 1951 für *Cyclops*-Eier). Der Kernsaft der Interphasekerne war frei von cytochemisch erfaßbarer Ribonukleinsäure. Es ist hier vielleicht ganz interessant, auf die zeitlichen Verschiedenheiten in der Ansammlung von Kohlenhydraten und Ribonukleinsäure — Stoffen, die offenbar während der Spindelbildung gebraucht werden — im Kernsaft von sich teilenden Kernen hinzuweisen.

Bevor aus oben angeführten Versuchen allgemein gültige Schlußfolgerungen über die Bedeutung der in Kern und Spindel vorkommenden Kohlenhydrate gezogen werden können, ist erst eine breitere Kenntnis über das Vorkommen der Kohlenhydrate notwendig.

Zusammenfassung.

Kerne verschiedener tierischer Zellen und der Alge *Acetabularia* wurden mit Hilfe der Hotchkiss-Reaktion auf ihren Kohlenhydratgehalt geprüft. Hierbei ergab sich, daß sich nicht teilende Kerne in Zellen mit einer starken Eiweißsynthese (Ganglienkerne von Mäusen und Tauben, Leberkerne von Mäusen, Oocytenkerne von *Cyclops*, *Diaptomus*, *Tipula* und *Ascaris*) frei von cytochemisch erfaßbaren Kohlenhydraten sind (ausgenommen *Acetabularia*), während in sich teilenden Kernen (Oogonien- und Furchungskernen von *Cyclops*) Kohlenhydrate nachgewiesen werden können. Die Kohlenhydrate erscheinen im Kernsaft und in der aus dem Kernsaft intranukleär sich ausbildenden Spindel, sowie in den Centrosomen und Astrosphären. Der Kohlenhydratgehalt der Kerne wechselt je nach Funktion dieser. So ist die Hotchkiss-Reaktion bei *Cyclops* positiv in Oogonienkernen, negativ in Oocytenkernen, positiv in Oocytenkernen kurz vor der Reifeteilung, positiv in Furchungskernen, negativ in den Urgeschlechtszellen, die sich während der Embryonalentwicklung nicht mehr teilen. Diese Befunde weisen auf eine wesentliche Bedeutung der Kohlenhydrate für den extrachromosomalen Teilungsapparat, wie Spindel, Centrosom und Astrosphäre hin.

Zusatz bei der Korrektur. Weitere Untersuchungen ergaben, daß in Spindel und Kernsaft von Furchungsstadien des Polychaeten *Sabellaria spinulosa* und der Teleostier *Clupea harengus* und *Gadus morrhua* Kohlenhydrate nachweisbar sind, während in den Spermatocyten der Tipulide *Pales crocata* die Hotchkiss-Reaktion zu schwach ausfiel, um eindeutige Aussagen zuzulassen.

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